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Meichroacidin Containing the Membrane Occupation and Recognition Nexus Motif Is Essential for Spermatozoa Morphogenesis*[S]

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Meichroacidin (MCA) is a highly hydrophilic protein that contains the membrane occupation and recognition nexus motif. MCA is expressed during the stages of spermatogenesis from pachytene spermatocytes to mature sperm development and is localized in the male meiotic metaphase chromosome and sperm flagellum. *MCA* sequences are highly conserved in *Ciona intestinalis*, *Cyprinus carpio*, and mammals. To investigate the physiological role of MCA, we generated *MCA*-disrupted mutant mice; homozygous *MCA* mutant males were infertile, but females were not. Sperm was rarely observed in the caput epididymidis of *MCA* mutant males. However, little to no difference was seen in testis mass between wild-type and mutant mice. During sperm morphogenesis, elongated spermatids had retarded flagellum formation and might increase phagocytosis by Sertoli cells. Immunohistochemical analysis revealed that MCA interacts with proteins located on the outer dense fibers of the flagellum. The testicular sperm of *MCA* mutant mice was capable of fertilizing eggs successfully via intracytoplasmic sperm injection and generated healthy progeny. Our results suggest that MCA is essential for sperm flagellum formation and the production of functional sperm.

Haploid cells differentiate only during spermiogenesis following meiosis. The specific features of spermiogenesis include the formation of the tail, mitochondria, and acrosome, nuclear condensation, and the elimination of spermatid cytoplasm. To understand haploid germ cell differentiation fully, *i.e.* spermiogenesis, it is important to identify and characterize specific genes expressed during these developmental processes (1). Meichroacidin (MCA,[3] male meiotic metaphase chromosome-associated acidic protein) was originally isolated using polyclonal antibodies against testicular antigens (2) and is expressed in male germ cells and expressed weakly in the mouse ovary. Specifically, MCA protein is localized predominantly in the cytoplasm of cells throughout the stages of sperm development, *i.e.* from pachytene spermatocytes to round spermatids, as well as in the regions of metaphase chromosomes and spindles during both the first and second meiotic divisions (2). The amino acid sequence of MCA contains a set of seven nominal repeat sequences consisting of the repeat sequence *YXGXX(X)XXXX*-HGQG (2). Recently, junctophilins (JPs), which are a novel conserved family of proteins that are components of the junctional complexes, were reported to contain a repeat amino acid sequence similar to that of MCA (3). However, MCA consists of a hydrophilic amino acid region, whereas JPs have a C-terminal hydrophobic segment spanning the endoplasmic/sarcoplasmic reticulum and are expressed abundantly in a variety of tissues (3, 4). The repeat amino acid sequence, referred to as the membrane occupation and recognition nexus (MORN) motif, is a novel protein-folding module that is shared by functionally different proteins and may have specific physiological roles (3). Southern blots revealed positive bands hybridizing to mouse

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S7 and Tables S1 and S2.

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[3] The abbreviations used are: MCA, meichroacidin; MORN, membrane occupation and recognition nexus; JP, junctophilin; SNP, single nucleotide polymorphism; ODF, outer dense fiber; TUNEL, deoxynucleotidyltransferase-mediated dUTP nick end-labeling; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; CTAB, cetyltrimethylammonium bromide; PFA, paraformaldehyde; FS/H, fibrous sheath/head; h, human; Ax, axoneme.

MCA cDNA probes in chromosomal DNA samples from other species, including rats, chickens, *Xenopus*, pufferfish, and humans (2). Recently, the *MCA* homolog of the carp (*Cyprinus carpio*) MORN motif-containing sperm-specific axonemal protein (MSAP) was cloned and characterized (5). *MCA* homologs have been found in organisms ranging from unicellular green algae to mammals through the use of computer-assisted analysis (5). In carp and ascidians (*Ciona intestinalis*), *MSAP* is expressed during late spermatogenesis and accumulates in mature spermatozoa, where it is localized in the basal body and flagellum (5, 6). The isolation and characterization of human *MCA* (*h-MCA*) indicate that h-MCA protein is localized in the sperm flagellum and basal body (7, 8). These results suggest that h-MCA plays an important physiological role in flagellum formation during spermiogenesis.

Here we demonstrated that MCA is expressed in cytoplasm spermatids and is dominantly localized in the outer dense fibers of the flagellum. In addition, mice deficient in MCA exhibit male infertility and azoospermia because of impaired sperm formation. We used single nucleotide polymorphism (SNP) studies to identify two SNPs that induce amino acid substitutions in azoospermic or oligospermic infertile human males. It is possible that SNPs in MCA are related to human infertility.

EXPERIMENTAL PROCEDURES

Animals—All mice were bred and maintained in our laboratory animal facilities and used in accordance with the guidelines for the care and use of laboratory animals set forth by the Japanese Association for Laboratory Animal Science. The mice were kept under controlled temperature and lighting conditions throughout the experiments and were provided with food and water *ad libitum*.

Northern Blots—Total RNA was isolated from various mouse tissues using RNA zolTM B (Invitrogen). Total RNA was extracted according to the manufacturer's instructions and was quantified using optical density measurements. RNA samples containing 2.2 M formaldehyde were electrophoresed in 1.1% agarose gels containing 0.66 M formaldehyde. The RNA was transferred to a nitrocellulose filter in 20× SSC and hybridized with ^{32}P-labeled cDNA prepared using the BcaBest random primer kit (Takara, Shiga, Japan) at 65 °C for 2 h in a PerfectHyb (Toyobo, Osaka, Japan).

Western Blots—Testes freshly removed from mice were homogenized on ice with TBS-T buffer (100 mmol/liter Tris-HCl, pH 7.5, 150 mmol/liter NaCl, 0.2% Tween 20). After centrifugation at 17,800 × *g*, the protein concentration of the supernatant was estimated using a Bradford protein assay (Nacalai, Kyoto, Japan). Following protein quantification, 50 μg of protein from each extract were subjected to SDS-PAGE, followed by electroblotting to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membranes were blocked with 5% nonfat dry milk and washed for 15 min with TBS (100 mmol/liter Tris-HCl, pH 7.5, 150 mmol/liter NaCl). They were then incubated with each antibody overnight at 4 °C and washed in TBS once for 3 min and then three times for 5 min each. Finally, the membranes were incubated with peroxidase-conjugated anti-rat or anti-rabbit immunoglobulins (1:500; Amersham Biosciences) for 2 h at 25 °C. After further washing, the reactive bands were visualized on development with the POD staining kit (Wako, Osaka, Japan).

Immunoprecipitation—Testicular fractions were lysed with RIPA (10 mmol/liter Tris-HCl, pH 7.5, 150 mmol/liter NaCl, 0.1% deoxycholic acid, 0.3% SDS, 1% Nonidet P-40, and 0.5 ml/liter protease inhibitor mixture; Nacalai). Each antibody was added to Dynabeads-protein G (Invitrogen), washed once with PBS, and then washed three times with PBS containing 0.01% Tween 20 as per the manufacturer's recommendations. The lysates were then centrifuged at 10,000 rpm for 10 min at 4 °C. Glycerol was added to the supernatants at a concentration of 10%. The Dynabeads-protein G was treated with the lysates at 4 °C overnight and was then washed with PBS containing 10% glycerol and 0.1% Tween 20. SDS sample buffer was added to the Dynabeads-protein G, and each sample was subjected to Western blotting.

Mouse Sperm Protein Fractionation—Sperm was collected from 10 mice using Percoll (9) and was fractionated according to a previously described adapted protocol (10). Briefly, sperm was washed from the epididymis and vas deferens and subjected to three sequential extractions at 4 °C in 500 μl of a solution containing 1% Triton X-100 and 2 mM dithiothreitol in 50 mM sodium borate buffer at pH 9.0 for 40 min each. After each extraction, the samples were centrifuged at 400 × *g* using a Tomy MRX-150 centrifuge (Tomy, Tokyo, Japan), and the supernatants were collected (membrane soluble fractions: M1, M2, and M3). The pellet was washed three times with 50 mM sodium borate buffer and suspended in 500 μl of a solution of 0.6 M potassium thiocyanate (KSCN), 2 mM dithiothreitol, and 50 mM Tris-HCl, pH 8.0, for 2 h at 4 °C. After centrifugation at 800 × *g*, the supernatant was collected (central axoneme (Ax) fraction), and the pellet was extracted overnight at 4 °C in 500 μl of a solution containing 4 M urea, 50 mM Tris-HCl, pH 8.0, and 2 mM dithiothreitol. A final centrifugation at 17,800 × *g* was performed to separate the urea-extracted fraction (urea fraction, ODF). Finally, the resulting nonextracted pellet was washed in borate buffer three times, suspended in sperm extraction buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 5% mercaptoethanol), and sonicated on ice for 10 min (fibrous sheath/head fraction: FS/H). The protein concentration of each fraction (*i.e.* M1, M2, M3, Ax, ODF, and FS/H) was estimated using the Bradford protein assay (Nacalai). Fractions M3, Ax, and ODF were precipitated with 10% trichloroacetic acid. Approximately 20 μg of protein from each fraction was separated by SDS-PAGE in 10% polyacrylamide gels. Western blotting was performed as described above. Control antibodies for the membrane (anti-Izumo1) (11), FS/H (anti-AKAP82) (12), and ODF (anti-Odf1) fractions (13) at a final dilution of 500× were used to verify the extracted proteins.

Immunohistochemistry of Sperm—Mouse sperm from the vas deferens and caudal epididymis suspended in PBS was filtered through a nylon mesh and centrifuged at 400 × *g*. The pellet was then washed in PBS, and a few drops were placed on glass slides and dried at 55 °C for 10 min. The slides were blocked with 10% blocking solution (Nacalai) whole serum in PBS for 30 min at room temperature. The drying and blocking conditions were kept the same for all immunohistochemical procedures. Blocked samples were incubated with anti-MCA antibodies (10

μg/μl IgG) or preimmune serum IgG, both diluted in PBS (1000×), overnight at 4 °C. After one wash, the slides were treated with diluted (1000×) anti-rabbit IgG goat serum conjugated with rhodamine or fluorescein isothiocyanate (FITC) for 1 h at room temperature. The slides were then washed and examined under a fluorescence microscope. Triton X-100- and urea-treated sperm obtained from the fractionation of sperm proteins were also examined immunohistochemically using the same protocol. To visualize individual fibers of the ODF, mature sperm was treated for 1 h at room temperature with a solution containing 10 mM Tris-HCl, 30 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, and 0.05% cetyltrimethylammonium bromide (CTAB), which is a cationic detergent that, under reducing conditions, extracts all tail structures except for the Odfs, which are released from the tight native form (14). Co-localization of MCA with Odf2 protein was accomplished by incubating intact and CTAB-treated sperm with anti-MCA antibodies that had been conjugated to rhodamine with an EZ-label rhodamine protein labeling kit (Pierce) for 1 h at room temperature. After a thorough wash in PBS, the samples were incubated with anti-Odf2 rabbit polyclonal antibodies for 2 h at room temperature, washed in PBS, and treated with FITC-conjugated diluted rabbit IgG antibody (2000×) for 1 h at room temperature.

Generation of MCA Targeting Mouse—The *MCA*-targeting construct was created by PCR amplification of a homologous 4-kb 5′ arm and 1-kb 3′ arm using *129Sv* genomic DNA as the template. Targeting at the *MCA* genomic locus resulted in the replacement of exons 1–3 with a neomycin cassette. Two amplified fragments were ligated sequentially into cloning sites on either side of the neomycin resistance gene in the targeting vector backbone. The targeting vector contained the neomycin resistance gene and a thymidine kinase gene, both under the control of the PGK promoter. The vector plasmid was linearized by NotI digestion before electroporation into W9.5 embryonic stem cells. Of 144 G418 gancyclovir-resistant clones that were screened for the targeting event, Southern blotting showed that two had undergone the correct homologous recombination. The two targeted cell lines were injected into C57BL/6J blastocysts, resulting in the birth of male chimeric mice. Highly chimeric males were mated with C57BL/6J wild-type females to generate F_1 offspring, half of which were heterozygous for the targeted allele. The embryonic stem cell lines were injected and produced a high percentage of chimeras that entered the germ line. Heterozygous F_1 males were then crossed with C57BL/6J females to obtain heterozygous F_2 animals. Heterozygous F_2 animals were bred to obtain homozygous mutants or to check the Mendelian inheritance. Eight mice older than 3 months were used to determine the fertility rate. The phenotypic variation was assessed, and biochemical analyses were conducted on samples from at least six individuals.

Southern Blotting and PCR—Genomic DNA was extracted from mouse tails using standard protocols (15). Southern blots were conducted to determine the site of the integration of the gene trap sequence in the *MCA* gene locus and to genotype the mice. A 700-bp probe for genomic Southern blotting was generated by PCR amplification from mouse genomic DNA. Genomic DNA samples (10 μg) were digested with ScaI and electrophoresed in 0.8% agarose gels. Southern hybridizations were carried out using standard protocols (15). The mice were genotyped by PCR using two sets of primers (see supplemental Fig. S1) as follows: one set of primers (5′-GGAGTAGCAAGTGATGTCAGGTCC-3′ and 5′-GAGTAACCTGAGGCTATGGCAGG-3′) to amplify the *Neo* gene and one set of primers (5′-CTATCAAGCAGTTACCAGCCACCC-3′ and 5′-GCAGAGGGAGCGAGGCTCAGCACATGG-3′) for the *MCA* gene.

Morphological and Immunohistochemical Observations—For the histochemical examination, fresh testis samples were embedded in O.T.C. compound embedding medium (Sakura Finetek, Tokyo, Japan) and frozen at −20 °C. Then 8-μm thick sections were prepared using a cryomicrotome (HM 500 OM; Microm, Walldorf, Germany) and fixed with 4% paraformaldehyde (PFA) at 4 °C for 10 min. Alternatively, testis samples were fixed in Bouin's solution for 24 h. After fixation, the samples were embedded in paraffin and sectioned at 8-μm thickness. Deparaffinized sections and frozen sections were incubated with the anti-MCA antibody (10 μg/μl IgG) or preimmune serum IgG, both diluted in PBS (1000×) overnight at 4 °C. After one wash, the slides were treated with diluted (1000×) anti-rabbit IgG goat serum conjugated with horseradish peroxidase for 1 h at room temperature. The slides were then washed and visualized by development with the POD immunostain kit (Wako, Osaka, Japan). Sections were treated with each antibody or stained with hematoxylin and eosin. Morphological identification of spermatogenic cells was based on the criteria of Russell *et al.* (16). The number of seminiferous tubules that sloughed spermatocytes or spermatids into the lumen was counted for 35 tubules of heterozygous or homozygous mutant mice.

For electron microscopy, the testis was perfused with 3% glutaraldehyde in HEPES buffer (10 mM HEPES and 145 mM NaCl). After post-fixation with 1% osmium tetroxide, the testis was embedded in Epon. Selected areas were then sectioned and examined.

Detection of Apoptosis—To identify apoptotic cells, we performed immunostaining with anti-active caspase 3 antibody (Promega, Madison, WI) and terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining using an *in situ* apoptosis detection kit (Takara) according to the manufacturer's instructions. The number of TUNEL-positive signals was counted for 20 seminiferous tubules of heterozygous or homozygous mutant mice. To identify Sertoli cells, we used anti-GATA 4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). For immunostaining by anti-active caspase 3 and GATA 4, sections of testis fixed in 4% PFA were treated with an antigen unmasking solution (Vector Laboratories, Burlingame, CA). Immunostaining was performed according to the manufacturer's instructions.

Testicular Sperm Extraction with Intracytoplasmic Sperm Injection (TESE-ICSI)—Testis was placed in cold HEPES-CZB, and the tunica albuginea was removed. The bundles of seminiferous tubules were carefully separated using forceps. The separated tubules were examined under a dissecting microscope. Mature sperm was squeezed out from regions of the tubules that were darkened in the innermost part using forceps (17, 18).

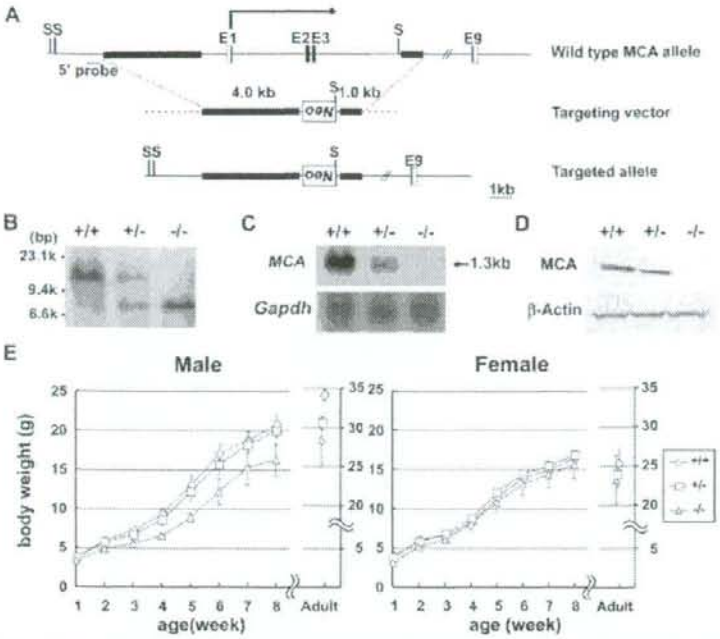

FIGURE 1. **Generation of *MCA* knock-out mice.** *A*, schematic representation of the methods used for gene targeting of the *MCA* genome. The gene targeting construct contains the *Neo* gene (*open box*) between the 4-kb 5′ arm and the 1-kb 3′ arm (*thick lines*). As a result, the regions of exons 1–3 were replaced by the *Neo* gene. Exon 1 includes the first methionine. *Arrows* indicate the transcriptional direction of *MCA*. *S* indicates ScaI restriction sites. *B*, targeted allele was identified by Southern blotting of genomic DNA digested with ScaI using a DNA probe created from the 5′ fragment. *C*, Northern blotting to localize gene expression. *MCA* transcripts are not detectable in the testis of *MCA* homozygous mice. The same membrane was re-hybridized with *Gapdh* cDNA as a control. *D*, Western blotting of testicular lysates of adult mice using an anti-MCA polyclonal antibody. The MCA protein is not detected in the testicular lysate of the homozygous mouse. β-Actin was used as a control. *E*, growth curve of male and female offspring from heterozygous matings (male +/+, $n = 4$; male +/−, $n = 9$; male −/−; $n = 4$; female +/+ l $n = 7$; male +/−, $n = 11$; female −/−, $n = 7$).

The sperm was suspended in 12% polyvinyl pyrrolidone in HEPES-CZB and injected into the cytoplasm of unfertilized eggs using a piezo-driven micromanipulator (Prime Tech, Ibaraki, Japan) within 1 h of preparation (19, 20). The injected eggs were cultivated in kSOM (20) overnight and then transferred to the oviducts of pseudopregnant females.

Serum Testosterone—Serum testosterone was measured using an enzyme immunoassay (testosterone enzyme-linked immunosorbent assay kit, catalog number 1880, Alpha Diagnostics, San Antonio, TX).

Identification of SNPs in the Open Reading Frame of MCA—Infertile patients ($n = 245$) were divided into subgroups according to the degree of defective spermatogenesis (21). Of these patients, 153 (68%) had nonobstructive azoospermia and 73 (32%) had severe oligospermia ($<5 \times 10^6$ cells/ml). The control group of fertile males ($n = 172$) included men who had fathered children born at the maternity clinic. DNA samples were extracted from the blood leukocytes of the infertile and proven-fertile males. Genomic DNA was isolated from the blood samples using protease and phenol purification (15). Eight PCR primer sets were designed to amplify the exons of *MCA* genes (see supplemental Tables S1 and S2 and supplemental Fig. S2). PCR was performed using PrimeSTAR or EX Taq hot start (Takara) (see supplemental Table S2). The PCR-amplified fragments were purified using CleanSEQ (Beckman Coulter, Tokyo, Japan), and thermal cycle sequencing (Applied Biosystems, Foster City, CA) was performed. The DNA sequences were determined using the same PCR primers.

Statistical Analysis—Differences between the experimental and control conditions were compared using one-way analysis of variance with Fisher's protected least significant difference tests. Significant differences ($p < 0.05$) are discussed.

RESULTS

MCA Homozygous Mutant Males Are Azoospermic—To investigate the physiological role of MCA, we generated homozygous *MCA* knock-out mice. We constructed the targeting vector (Fig. 1*A*), and homologous recombination was used to generate embryonic stem cell clones that were heterozygous for the *MCA* mutation. To produce chimeric mice, transgenic embryonic stem cells were injected into blastocysts that were subsequently implanted into pseudopregnant mice. We performed Southern blotting to confirm correct recombination (Fig. 1*B*). *MCA* mRNA was not detectable using Northern blots (Fig. 1*C*), and the 40-kDa MCA protein was not detectable using Western blots (Fig. 1*D*) of the testis of homozygous *MCA* mutant mice. Crosses of heterozygous mutant pairs produced the expected ratios of wild-type, heterozygous, and homozygous genotype offspring, according to classical Mendelian inheritance patterns. Homozygous mutant males had slightly smaller body masses than did wild-type males (Fig. 1*E*). Matings between homozygous *MCA* knock-out males and wild-type females did not produce any successful pregnancies during a period of more than 3 months of continuous cohabitation, although vaginal plugs were observed in the paired wild-type females (Table 1). The heterozygous male *MCA* mutant mice and the homozygous females were all fertile (Table 1). Female body mass (Fig. 1*E*), newborn pup growth rates (Fig. 1*E*), and the weights of various organs, including the testes and seminal vesicles of adult *MCA* homozygous mutant mice, did not differ significantly from those of wild-type mice (Table 2). The serum testosterone levels of adult *MCA* homozygous mutant mice were normal compared with those of wild-type mice (Table 2).

MCA Is Essential in Sperm Formation—To confirm the inactivation of *MCA*, an immunohistochemical examination was

TABLE 1
Fertility rates of mutant mice

Genotype	Male fertility (no. of fertile males/no. of vaginal plugs)	Litter size (average no. of newborn pups)[a]	Female fertility (no. of fertile females/no. of vaginal plugs)	Litter size (average no. of newborn pups)[a]
+/−	10/10	8.2 ± 0.3	10/10	7.8 ± 0.3
−/−	0/21	0	8/8	6.0 ± 0.4

[a] Values are means ± S.E.

TABLE 2
Weights of organs, serum testosterone levels, and characteristics of seminiferous tubules in mutant mice
All values are means ± S.E. $n = 6$ mice per genotype.

Parameter	Value for $MCA^{-/-}$ mice	Value for $MCA^{+/-}$ mice	Value for $MCA^{+/+}$ mice
Wet weight of organs/body weight			
Testis	3.3 ± 0.2	3.5 ± 0.2	2.9 ± 0.2
Epididymis	1.5 ± 0.2	1.6 ± 0.1	1.5 ± 0.1
Seminal vesicle	12.7 ± 1.23	12.66 ± 1.45	13.47 ± 1.45
Testosterone level (ng/ml)	1.025 ± 0.730	1.305 ± 1.078	0.917 ± 0.622
Diameter of seminiferous tubules (μm, $n = 8$ tubules per genotype)	165.19 ± 5.82	168.83 ± 4.75	177.14 ± 4.33
No. of cells in seminiferous tubules ($n = 8$ tubules per genotype)	407.8 ± 15.6	421.5 ± 21.6	423.8 ± 15.9

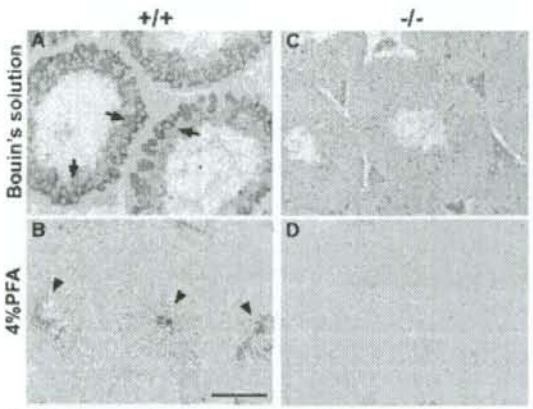

FIGURE 2. **Immunohistochemical staining of the cross-sections of mouse testis with anti-MCA antibody.** The cross-sections fixed in each solution (*left margin*) were incubated with anti-MCA antibody. Cross-sections of wild-type testis (*A* and *B*) or mutant testis (*C* and *D*) are shown. The signals of the cytoplasm of spermatocytes (*arrow*) (*A*) and sperm flagellum (*arrowheads*) (*B*) disappeared in cross-sections of mutant mice (*C* and *D*). *Bar* = 100 μm.

performed. The anti-MCA polyclonal antibody stained some germ cells in testis preserved in Bouin's solution (Fig. 2*A*) (2). The signal was detected in the spermatocytes and elongated spermatids (Fig. 2*A*), and these signals decreased gradually as morphogenesis proceeded, as reported by Tsuchida *et al.* (2). The heat treatment during embedding with paraffin or chemical bonds with the fixative solutions may have altered the exposed sites of the proteins to which the antibodies bound and may have caused the difference in immune staining. To examine the expression of MCA precisely, we performed immunohistochemical analyses using cross-sections preserved in 4% PFA (Fig. 2*B*). The anti-MCA antibody predominantly stained flagella in sperm preserved in 4% PFA (Fig. 2*B*). In *MCA* homozygous mutant males, the signals from testis preserved in either Bouin's solution or 4% PFA disappeared (Fig. 2, *C* and *D*). Rabbit IgG antibody was used as a control (see supplemental Fig. S3). These results indicate that the signals with both types of fixation depend on the *MCA* gene products. In addition, MCA was expressed from the pachytene spermatocyte (stage V) to sperm stages. These observations are in agreement with previous expression profile analyses using Northern and Western blotting (2). MCA might change the conformation in each subcellular localization and play different roles in sperm flagella and the cytoplasm of spermatocytes and spermatids; thus, the difference in conformation might cause different signals for each fixation method.

On microscopic examination, the diameter of the seminiferous tubules and number of cells within the testes of adult wild-type and *MCA* homozygous mutant mice did not differ significantly (Table 2). There were also no significant differences in testis mass between mutant and wild-type mice (Table 2). The spermatogonia, spermatocytes, and spermatids were arranged systematically in the seminiferous tubules in heterozygous mutant testes, just as in wild-type testes; the spermatogonia are located in the tubule walls, and the spermatids are located at the tubule centers, and spermatocytes are located between the two (Fig. 3, *A* and *B*). In mice, the spermatogenic cycle that occurs in each tubule of the seminiferous epithelium is divided into 12 stages (16). In addition, germ cells in the seminiferous tubules are enclosed by Sertoli cells. The spermatocytes or round spermatids had peeled from the Sertoli cells and were found at the tubule centers in ~6% of the seminiferous tubules of mutant testes. Therefore, the arrangement of germ cells in cross-sections was disturbed in *MCA* homozygous mutant testis tubules compared with *MCA* heterozygous mutant testis and wild-type tubules (Fig. 3, *E* and *F*). The epididymides of heterozygous mutant mice were filled with sperm (Fig. 3, *G* and *I*), whereas sperm were sparse or absent in the epididymides of homozygous mutant mice, as observed using light microscopy (Fig. 3, *H* and *J*). *MCA* mutant sperm that was present in the testes had few abnormalities and almost normal head shapes (Fig. 3, *K–P*).

Using electron microscopy, almost all sperm in homozygous *MCA* mutant testes showed abnormalities (Fig. 4 and supplemental Fig. S4), although developed sperm was observed in the seminiferous tubules using light microscopy, and there was no difference in the weights of mutant and wild-type testes (Fig. 3*D* and Table 2). Electron microscopy showed that most spermatids developed normally to step 9 in homozygous mutants (data not shown). However, following nuclear condensation, the rearrangement of mitochondria, and flagellum formation, we observed that the construction of these parts was disturbed, and small vacuoles appeared during spermatid elongation (Fig. 4*A*). Furthermore, the elongated spermatids in homozygous mutant testis were phagocytosed by Sertoli cells (Fig. 4*B* and supple-

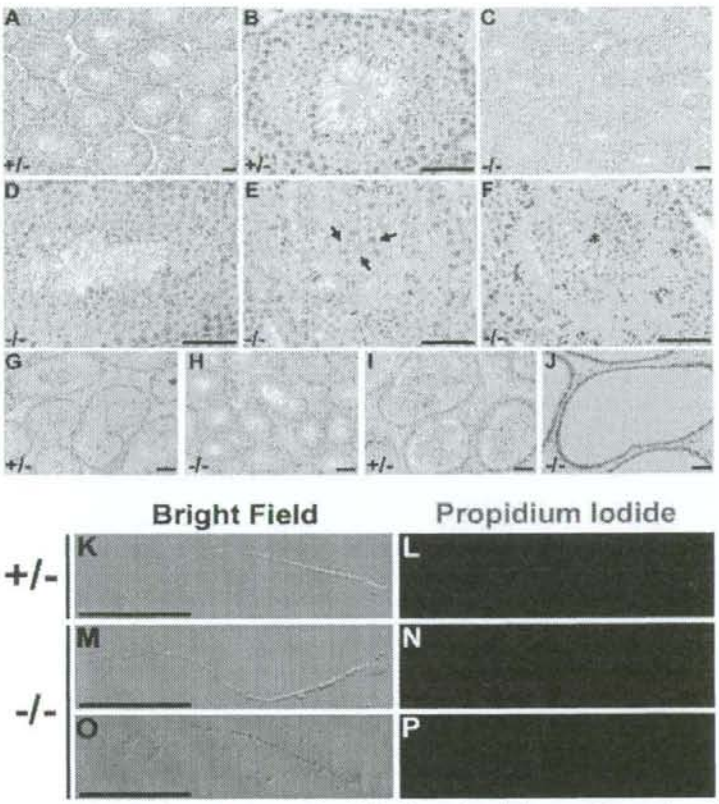

FIGURE 3. **Histological analyses of mutant testis, epididymides, and testicular sperm.** Cross-sections of heterozygous (*A* and *B*) or homozygous mutant (*C–F*) testis are shown. *B* and *D–F* show testicular tubules under high magnification. *Arrows* and an *asterisk* indicate irregularly arranged spermatocytes and round spermatids, respectively. Cross-sections of heterozygous (*G* and *I*) or homozygous mutant (*H* and *J*) epididymides are shown. *G* and *H* show caput epididymides and *I* and *J* show cauda epididymides. Sperm was not found in homozygous mutant epididymides (*H* and *J*). Heterozygous (*K* and *L*) and homozygous mutant (*M–P*) testicular sperm were observed. The heads of the homozygous mutant sperm have some abnormal features (*O* and *P*). *Bar* = 50 μm.

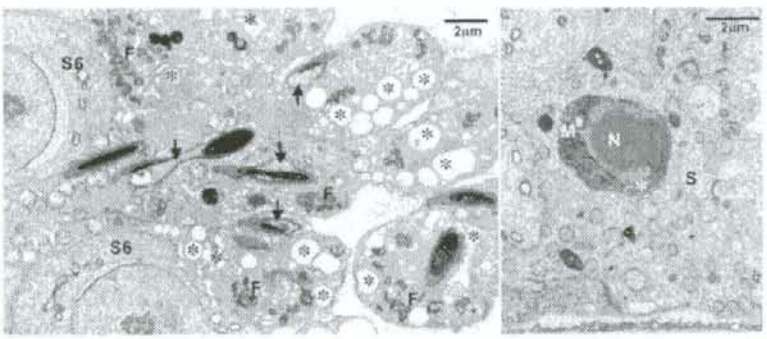

FIGURE 4. **Electron micrographs showing the degeneration of spermatids.** Sections of *MCA* homozygous mutant testis are shown. *A*, spermatids in a stage VI seminiferous tubule. Step 15 spermatids show deformation of the heads (*arrow*) and numerous vacuoles (*) in the cytoplasm. Flagella (*F*) that contain an axoneme, mitochondria, outer dense fibers, and fibrous sheaths are formed; however, these components are disarranged in the cytoplasm. Step 6 spermatids (*S6*) appear to be normal in shape. *B*, spermatid phagocytosed by Sertoli cells (*S*). The highly deformed nucleus/head (*N*), acrosome (*), and mitochondrial sheath (*M*) of the spermatids are shown.

mental Fig. S4). These findings indicate that the absence of *MCA* expression leads to abnormalities in spermatid formation, which in turn results in the abnormal morphogenesis of other components of the sperm. Alternatively, the MCA protein may affect various aspects of sperm morphogenesis directly.

MCA Was Strongly Associated with the ODF—Western blots of the subcellular fractions of sperm proteins indicated that MCA expression was present predominantly in the flagellum and more weakly in the sperm head (Fig. 5*A*). We also performed subcellular fractionation of sperm proteins located in the membrane/cytoplasm, axoneme, ODF, and FS/H. Soluble fractions and the final FS/H-insoluble fractions were separated by SDS-PAGE, transferred to a membrane, and subjected to Western blotting with anti-MCA antibody. MCA was weakly eluted in the membrane fraction that solubilized with a nonionic detergent (Triton X-100) but was mostly solubilized with potassium thiocyanate and urea (Fig. 5*B*). The majority of MCA was indeed recovered in the axoneme and ODF fraction, although a small amount of MCA remained in the nonextracted pellet that contained FS/H fractions (Fig. 5*B*). Izumo sperm-egg fusion 1 (Izumo1) (11) and AKAP82, the major protein of the fibrous sheath of the sperm flagellum (12), were present in the membrane and FS fractions, respectively. Odf1, the major ODF protein (13), was extracted only with urea (Fig. 5*B*). MCA was extracted together with the cytoskeletal elements that make up the axoneme, ODF, and FS.

To examine MCA-protein complexes, immunoprecipitation was performed. In immunoprecipitating complexes of testicular lysate with anti-Odf1, Odf2, and SHIPPO 1/Odf3, MCA co-precipitated with anti-Odf1 and Odf2 (Fig. 5*C*). Furthermore, we examined protein complexes with Septin 4 and Septin 7 (Fig. 5*C*) (22). No signals were detected in protein complexes with these annulus proteins. In addition,

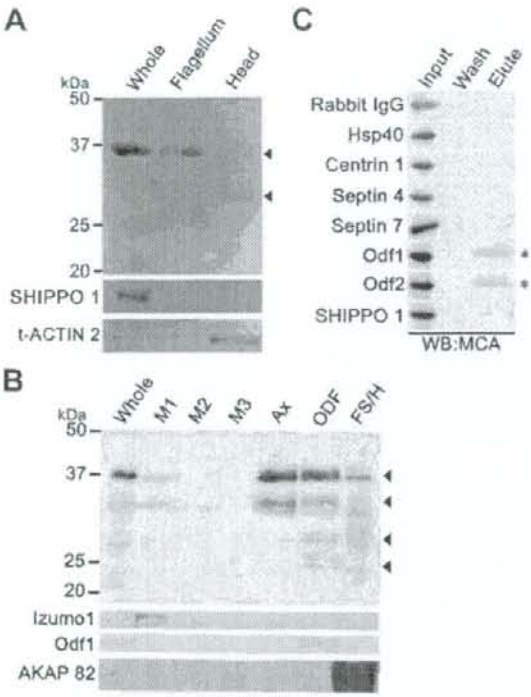

FIGURE 5. **Western blotting of the subcellular fractionation of sperm.** *A*, sperm was sonicated in PBS and fractionated by centrifugation. The *numbers* in the *left margin* indicate the molecular weights of the marker proteins. Anti-SHIPPO 1 or t-ACTIN 2 antibodies were used as controls in the flagellum or sperm head fractions (24, 37). *Arrowheads* indicate the signal of MCA. *B*, whole sperm fractions extracted with SEB. Approximately 20 μg of solubilized protein in each of the fractions obtained by sequential extraction with 1% Triton X-100 (*M1*, *M2*, and *M3*), potassium thiocyanate (*Ax*), and urea (*ODF*), and the remaining pellet (*FS/H*) extracted with SEB were tested for the presence of a sperm membrane protein (*Izumo1*), an FS component (*AKAP 82*), and an ODF protein (*Odf1*). Molecular weight marker bands are shown at the *left*. *Arrowheads* indicate the signal of MCA. *C*, lysate of testicular germ cells was immunoprecipitated with antibodies against flagellar proteins. Each antibody is indicated in the *left margin*. The Dynabeads-protein G treated with each antibody was reacted with the lysate (*Input*). After washing (*Wash*), the protein complexes were eluted from the Dynabeads using elution buffer (*Elute*). Each fraction was subjected to SDS-PAGE and Western blotting (*WB*) with anti-MCA antibodies. The *asterisk* indicates significant signals.

MCA was not detected in immunocomplexes with Hsp40 flagellum protein (6) and testis-specific centriole protein (*Centrin 1*, Fig. 5*C*) (23).

To examine the subcellular localization of MCA in detail, its presence in sperm treated with 0.05% CTAB and in samples from fractionation experiments following 1% Triton X-100 and 4 M urea treatment was determined using immunocytochemistry. Nontreated sperm displayed basic staining patterns for MCA (Fig. 6, *A–C* and see supplemental Fig. S5); the entire length of the sperm flagellum was stained homogeneously, and the sperm heads were stained weakly. In sperm treated with 1% Triton X-100, the membranes, organelles (including the mitochondrial sheath in the midsection), and cytosolic proteins are solubilized (24). These changes cause the sperm heads to bend because of the weakened support of the neck. In addition, the ODFs are sometimes observed to protrude from the weakened annulus region (10). In Triton X-100-treated sperm, the MCA signal was observed in the fibers along the rest of the flagellum and disappeared from the heads (Fig. 6, *D–H*, and see supplemental Fig. S5 in the supplemental materials). Therefore, MCA is associated with the ODF. As determined by fractionated Western blotting, the treatment of sperm with 4 M urea efficiently extracted most of the ODF protein together with MCA from the sperm flagellum. After only 15 min of exposure to urea, the MCA signal became scattered, and the fiber-like view of MCA in liberated ODF was lost (Fig. 6, *I–K*). Following 30 min of CTAB treatment, sperm released individual ODFs that were freed from the central core of the tail and dispersed distally to the connecting piece. Most sperm heads were separated from the tail, and resistant ODFs were frayed completely and joined together only at the connecting piece (14). MCA and Odf2 signals were clearly observed in individual fibers and the connecting piece (Fig. 6, *L–S*). Therefore, MCA is associated with ODFs and the connecting piece in a nonionic detergent-resistant form (Triton X-100). The MCA signal also appeared in the fibrous proteins that constitute the ODF, following exposure to an ionic detergent (CTAB).

Abnormal Sperm Might Be Phagocytosed by Sertoli Cells—The mass of *MCA* homozygous mutant testis was normal compared with that of wild-type mice (Table 2). However, there was no sperm in the epididymis of mutants. We also examined apoptotic cells in the testis. Apoptosis signals did not increase in germ cells, as determined by the detection of caspase-3 antibody (Fig. 7*A*). There were 0.33 ± 0.19 (average ± S.E.) and 0.20 ± 0.11 positive cells/tubule detected in the testes of homozygous and heterozygous mice, respectively. All cells in the centers of seminiferous tubules and Leydig cells were stained uniformly in testis sections of heterozygous and homozygous mutants. Therefore, these signals resulted from nonspecific staining. Using the TUNEL method, in the testes of heterozygous and homozygous mice, 0.15 ± 0.08 and 0.70 ± 0.19 TUNEL-positive cells/tubule, respectively, were detected (Fig. 7*B*). TUNEL-positive cells increased slightly in the germ cells of the homozygous mutant. There were 5.00 ± 0.75 strong signals with the shape of sperm nuclei per seminiferous tubule detected near the tubule walls, but not in the center, in the homozygous mutant mice only (Fig. 7*B*). The signal visualized using the TUNEL method was located near the nuclei of sperm in Sertoli cells (see supplemental Fig. S6). This signal may indicate the degraded products of sperm nuclei that were phagocytosed by Sertoli cells. *MCA* mutant sperm in the testis did not undergo apoptosis, but might be instead phagocytosed by Sertoli cells.

Sperm of MCA Mutant Mice Can Produce Viable Embryos—MCA was expressed from the spermatocyte through to sperm stages and was localized in meiotic metaphase chromosomes during meiosis (2). We examined whether the nuclei of *MCA* mutant sperm maintained the ability to produce viable embryos using testicular sperm extraction and intracytoplasmic sperm injection (TESE-ICSI). Sperm derived from heterozygous and homozygous *MCA* mutant mice was injected into eggs. Two-cell-stage embryos produced from heterozygous (n = 36) or

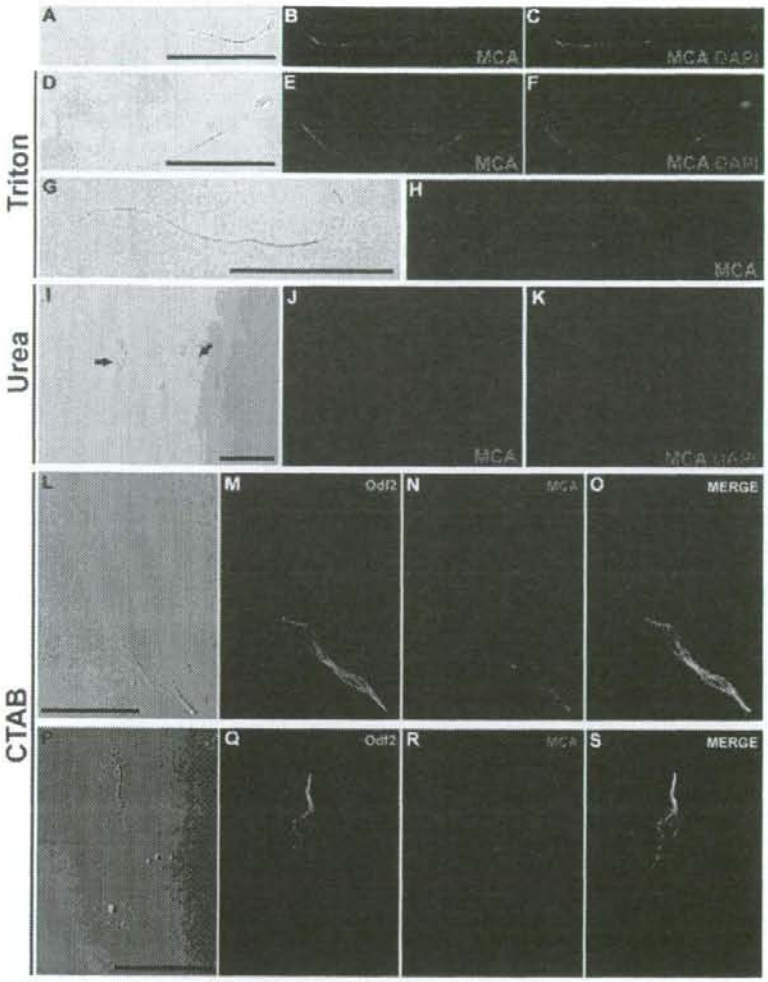

FIGURE 6. **Immunohistochemical examination of MCA in sperm.** Sperm and flagella from the cauda epididymis were stained with anti-MCA antibody (*A–C*). Following treatment with Triton X-100, the MCA signal disappeared from the sperm head but was maintained in the insoluble fractions of the flagellum (*D–H*). The signal disappeared from flagella subjected to urea treatment (*I–K*). Sperm treated with 0.05% CTAB to release the outer dense fibers (*L–S*) were stained with rhodamine-conjugated anti-MCA antibody (*N* and *R*). Samples were also reacted with anti-Odf2 antiserum and stained with FITC-conjugated secondary antibodies (*M* and *Q*). MCA signal was co-localized with Odf2 (*O* and *S*). *Bars* = 50 μm (*A–K*) or 25 μm (*L–S*).

homozygous ($n = 31$) *MCA* mutant sperm were transferred to the oviducts of pseudopregnant females. Nineteen (52%) and 10 (33%) pups were born from heterozygous and homozygous *MCA* mutant sperm, respectively, indicating that the nuclei of *MCA* mutant sperm were as capable of producing viable embryos as those of heterozygous sperm from fertile males.

SNPs in Human MCA—The analysis of human *MCA* sequences in more than 200 infertile male patients and in 172 proven-fertile male volunteers revealed two amino acid substitution-causing SNPs in the open reading frame of *MCA*. These SNPs were found in exon 2 in two of the azoospermia-infertile and in one of the oligospermia-infertile patients (Table 3 and see supplemental Fig. S2). The *a92c* SNP at amino acid 31, located within the first MORN motif in the N-terminal region, was found in three cases of heterozygosity and caused a shift from histidine to proline. The *g121a* SNP at amino acid 41, located between the first and second MORN motifs, was found in one case of heterozygosity and caused a shift from glycine to arginine. The *g393a* SNP in exon 5 at alanine 131 did not induce an amino acid substitution as seen by Rs2839536 in the dbSNP data base of the National Center for Biotechnology Information (NCBI). The prevalence of the *g393a* SNP was similar in proven fertile and infertile patients (Table 3).

DISCUSSION

In this study, we showed that the germ cell-specific *MCA* gene is essential for spermiogenesis and that two different SNPs in the human *MCA* gene (*h-MCA*) are associated with male infertility. A hydropathy plot (25) of the deduced MCA sequence revealed that MCA contains strongly hydrophilic regions throughout its total length (2). MCA is expressed predominantly in the cytoplasm of cells through a variety of stages, ranging from pachytene spermatocytes to sperm. MCA in sperm is localized throughout the cytoplasm and is specifically concentrated in the fibrous sheath (FS) and ODF of the flagellum. Apart from the axoneme and its associated proteins, the sperm flagellum consists of two exclusive cytoskeletal components as follows: the FS in the principal piece and the ODFs in the middle and principal pieces. Nine ODFs are anchored proximally at the connecting piece and run parallel to the tubulin doublets of the axoneme toward the distal end of the flagellum. Longitudinal columns and transversal ribs of the FS surround the ODF in the principal piece (26). The majority of proteins in the ODF and FS are hydrophobic and resist solubilization by ionic detergents (*e.g.* SDS) (10, 27, 28). Many proteins that were localized in this area have been identified previously (29). Of these proteins, glyceraldehyde-3-phosphate dehydrogenase-S was predominantly localized in the FS; however, it may be active only in the hydrophilic state

(30). There is little information available on how the hydrophilic FS and ODF are assembled in developing spermatids and how proteins in the FS and ODF maintain their functions. Even the hydrophilic protein MCA can occur in the FS and ODF, although it may be modified. Interactions between MCA and other proteins may support flagellum function and development.

MCA contains a set of seven MORN motifs. JPs, which are components of the junctional complexes that are expressed abundantly in the heart and brain, contain a conserved MORN motif (3, 4). The MORN motifs of JPs have tandem repeats of eight MORN motifs at the N-terminal region and a hydrophobic domain in the C-terminal region, which may function in anchoring the protein to the cellular membrane (see supplemental Fig. S7) (3). Other proteins containing a few MORN motifs have also been reported (see supplemental Fig. S7). MORN motifs may play an important role in protein complexes; however, the exact function of the MORN motif is not clear. The localization of the MORN motif in MCA was not significantly different from that in JPs. The exact function of the MORN motif may be more noticeable in MCA that consists of the MORN motif only.

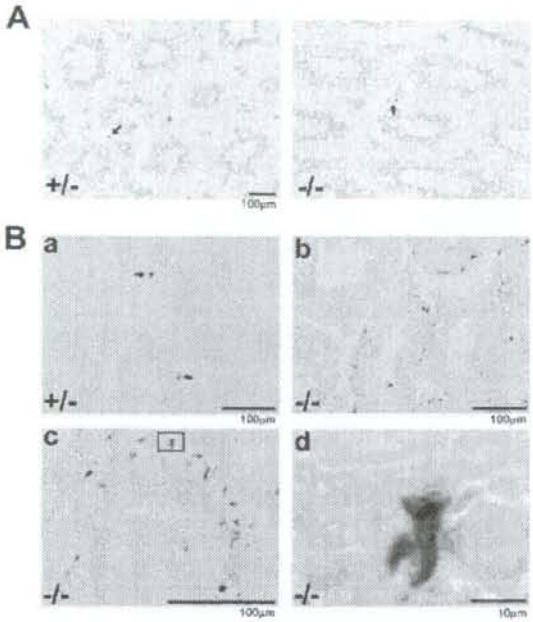

FIGURE 7. **Occurrence of apoptosis in the testis.** *A*, expression of active caspase 3 was observed in heterozygous (+/−) and homozygous (−/−) mutant mouse testis. *Arrows* indicate germ cells expressing active caspase 3. Cells in the centers of the tubules and Leydig cells were stained nonspecifically. *B*, TUNEL staining was performed on sections of testis. Apoptotic signals were observed in heterozygous mutant testis (*arrows*) (*panel a*). Signals indicated that phagocytosed cells were observed in the tubule walls of homozygous mutants (*panels b–d*). *Panels c* and *d*, high magnification of the signals found in mutant testis. *Panel d*, high magnification of the *box* in *panel c*.

Sperm in the testis of mutant mice appeared normal under light microscopy; however, detailed analysis using electron microscopy revealed some abnormalities. In other gene mutational mice that display abnormal sperm formation, sperm is sent to the epididymis even if it is abnormal (31). In addition, the abnormal sperm is observed in the vaginal plugs. However, the *MCA* mutant mice were azoospermic, and most defective sperm might be phagocytosed by the Sertoli cells. The absence of sperm in the epididimydis can be at least partly explained by Sertoli cell phagocytosis of defective sperms.

In the rat, epididymal ligation causes extensive degeneration of the seminiferous epithelium with loss of virtually the entire germ cell population and a significant decline in both testicular and epididymal weight (32), although ligation of the initial segment of the caput epididymis results in temporary testicular changes followed by evidence of recovery in the mouse (33). These testicular alterations differed from that of the *MCA* mutant testes. Furthermore, a few abnormally shaped sperm heads were occasionally observed in the cauda epididymis using light microscopy (data not shown). *MCA* mutant mice were nonobstructive azoospermic, although the relative size of the testis was normal. SNPs in the MORN motif of MCA only occurred in infertile men. These SNPs were heterozygotes of the major and minor SNP. *DMC1* is a *RecA* homolog that is specifically expressed during meiosis and is thought to play an important role. When one allele is not expressed in the DMC1 protein, the dysfunction does not appear (34). However, abnormal meiosis occurs when the mutant DMC protein is expressed from another allele (35). The male infertility-specific SNPs that we found may cause male infertility through their effects on mutant MCA protein expression. The *a92c* SNP may cause male infertility more readily than does the wild-type MCA and

TABLE 3
Prevalence of *MCA* SNPs in infertile and proven-fertile human populations

Exon	Position[a]			Genotype	No. of SNP				Reference (NCBI dbSNP)
	Nucleotide	Amino acid			Infertile		Proven fertile		
						%		%	
Exon 2	92	31	(H)	A/A	242	(99)	172	(100)	
			(H/P)	A/C	3	(1)	0	(0)	
			(P)	C/C	0	(0)	0	(0)	
	121	41	(G)	G/G	244	(99.5)	172	(100)	
			(G/R)	G/A	1	(0.5)	0	(0)	
			(R/R)	A/A	0	(0)	0	(0)	
Exon 5	393	131	(A)	G/G	141	(62)	82	(62)	
			(A)	G/A	79	(35)	41	(31)	Rs2839536
			(A)	A/A	7	(3)	9	(7)	
				ND[b]	20		40		

[a] Translation start site was +1.
[b] G393A haplotype in exon 5 was not decided exactly in 20 and 40 persons of infertile or proven fertile group, respectively.

may be transferred to the next generation via the female, as indicated by the results of the *MCA* mutant mouse study. Approximately 15% of couples that attempt to conceive over a 2-year period are unable to become pregnant (36). Recent technological developments in *in vitro* fertilization have ensured that even when sperm activity is low, pregnancy and birth are possible. The molecular mechanisms behind infertility remain uncertain. It is possible that SNPs in MCA are related to human infertility.

Collectively, our results demonstrate that MCA proteins containing the MORN motif play an important role in the construction of hydrophobic protein complexes in the sperm flagellum. These findings are valuable not only for understanding the molecular mechanisms of spermiogenesis but also for determining the function of proteins encoded by the MORN motif.

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Mechanisms of sperm-egg interactions emerging from gene-manipulated animals

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ABSTRACT Sperm-egg interactions have been studied for many years using biochemical approaches such as the employment of antibodies and ligands that interact with sperm or with eggs and their vestments. As a result, various factors that participate in fertilization have emerged. However, when animals were genetically manipulated to examine the roles of those factors, most of them were found, to our surprise, to be "not essential". Of course, all biological systems contain redundancies and compensatory mechanisms, but at least some factors were found to be "essential" after gene disruption. As a whole, the explanations of sperm-egg interactions require significant modification from the gene manipulation point of view. In this review, information about sperm-egg interactions obtained from genetically manipulated animals is mainly revisited in order to propose a new vision.

KEY WORDS: *fertilization, knockout mouse, sperm-zona interaction, sperm-egg fusion*

Introduction

About a billion years ago, living organisms devised sex (Margulis and Sagan, 1986) to facilitate a genetic shuffling for better survival and quick evolution. Since then enormous numbers of recognition and fusion of males and females have been successfully accomplished and the process has evolved immensely. In mammals, males produce innumerable tiny sperm while females produce much larger eggs with a thin glycoprotein layer: the zona pellucida. How do sperm and egg recognize each other, make contact, and achieve fusion? Various factors have been reported with convincing evidence. However, the recently-introduced, homologous gene disruption technique revealed many of the genes were dispensable because the animals devoid of those genes showed substantial fertilizing ability. One can argue that the disappearance of a factor could be compensated by adjusting the amount of equally functioning factors in gene disruption experiments. However, sperm are like "rockets" with limited resources available once launched. Sperm DNA are tightly packed with protamine and the transcriptions are shut down. Even if the sperm can carry out some *de novo* protein synthesis during capacitation using stored mRNA and mitochondrial ribosomes (Gur and Breitbart, 2006), it is difficult to imagine that the sperm rearrange their function by replacing one factor with others. One can also argue that, based on the importance of fertilization phenomena, various steps are made with backup systems. However, recent gene disruption experiments indicated that there are "essential" factors in fertilization, because in various cases, the lack of a factor leads to the complete loss of fertilizing ability of gametes. Thus the quest for more "essential" factors and analysis of the relationship among those factors represent the most promising path to elucidate the mechanism of fertilization in molecular bases. In the present review, we introduce essential factors in fertilization and clarify their relationships.

Eggs

Eggs are released into the peritoneal or bursal cavity and then are picked up by the cilia of the infundibulum. Mammalian eggs are almost invariably covered by numerous cumulus cells and an extensive extracellular matrix, which is recognized by specific receptors on the tips of the cilia. Pickup of this complex by the oviduct has been recorded in beautiful video pictures available online by Talbot *et al.* using hamsters (Talbot *et al.*, 1999) (Fig. 1).

After being picked up from the infundibulum, the eggs move to the ampulla of the oviduct and wait to be fertilized by sperm. It is not clear why or how they stay in this area, but they do, enveloped in the cumulus mass until fertilization occurs. Eggs have fertilizing ability depending on the nature of cumulus cells and zona pellucida (Yanagimachi, 1994), but surprisingly, fus-

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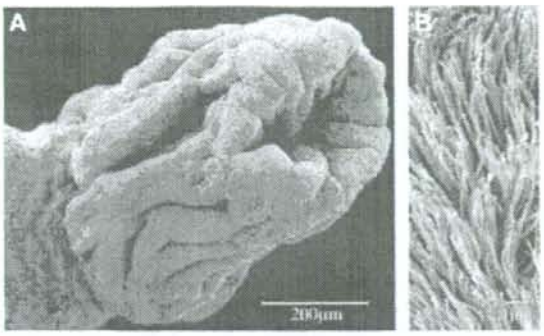

Fig. 1. Hamster infundibulum and magnified view of cilia on its surface *(Talbot* et al., *1999). An online video showing the movement of ovulated eggs into the oviduct is available at: http://www.molbiolcell.org/content/vol10/issue1/images/video/mk0190776002b.mov*

ing ability with sperm is formed even as - the immature eggs - develop without cumulus cells inside mouse testis (Isotani *et al.*, 2005).

Sperm

Sperm and eggs move in different directions. Sperm need to ascend the female reproductive tract to find eggs residing in the oviduct, while the eggs descend the oviduct and enter the uterus after fertilization. Usually one egg in humans and about ten in mice await sperm in the oviduct. Compared to the numbers of eggs, an overwhelming number of sperm are ejaculated into the female reproductive tract. However, only a small number of sperm reach the fertilization site. The uterus and oviduct are connected at the uterotubal junction (UTJ). The outer portion of the oviduct hangs into the uterus and forms a colliculus in mice, pigs and cows. The tract is very narrow at the UTJ and sperm are not able to migrate freely into the oviduct through the UTJ, decreasing the number of sperm participating in fertilization. It is not known if only selected sperm can penetrate the UTJ, but various factors are known to be essential for sperm to pass through the UTJ (Cho *et al.*, 1998, Hagaman *et al.*, 1998, Ikawa *et al.*, 2001, Nishimura *et al.*, 2004). When we made chimeric mice that ejaculated a mixture of wild-type sperm and motile but not fertile sperm from calmegin-disrupted mice, only the former sperm in the same ejaculates could migrate to the oviduct. This result indicates a possibility of selection at the UTJ (Nakanishi *et al.*, 2004).

Sperm chemotaxis toward eggs before fertilization has been demonstrated clearly in ascidians (Yoshida *et al.*, 2002). In humans, olfactory receptors (ORs) on sperm might function in finding eggs. The hOR17-4 was demonstrated to function in human sperm chemotaxis (Spehr *et al.*, 2003). Mouse sperm might also locate eggs by sensing a chemoattractant (Fukuda *et al.*, 2004). The disruption of an ion channel expressed in VNO neurons are reported to cause females to behave like males (Kimchi *et al.*, 2007). The contribution of ORs in fertilization must be proven by gene disruption experiments.

Sperm are produced in the testis, transferred into the epididymis and remain stored in the cauda epididymis. Once ejaculated, they must be activated by stimuli from the female environment and start to swim vigorously. Only capacitated and acrosome-reacted sperm are competent to fertilize eggs, like matches being struck and activated before acquiring capability to cause fire (Fig. 2)

The nature of the capacitation process is not well understood, but there are many papers indicating the importance of protein phosphorylation and calcium ion influx upon release of "decapacitation factor(s)" from sperm (De Jonge, 2005). It should be noted that the acrosome reaction is a change that happens only in capacitated spermatozoa and gradually increases to 30–40% during 1–2h of incubation *in vitro* in mouse sperm population. This means sperm are not homogeneous but are individually different from each other. However, most reports treat sperm as a mixed mass to evaluate sperm status because there is not a convenient method to separate sperm at different stages of capacitation and/or acrosome reaction. We must be aware that this homogeneity problem exists in the experiments which analyze sperm as a combined mass.

Various methods are proposed to observe acrosome reaction (Cross and Meizel, 1989, Larson and Miller, 1999, Saling and Storey, 1979). Our strategy to observe the acrosomal status under microscope is to use transgenic mouse lines with green fluorescent protein (GFP) in their acrosome. We produced transgenic mice whose sperm have GFP in their acrosome; the green fluorescence is clearly seen with no previous treatment of sperm. After the acrosome reaction, GFP disappears within three seconds (Nakanishi *et al.*, 1999). Sperm from these mice could easily be analyzed using a flow cytometer, and real-time analysis of acrosome reaction is possible (Nakanishi *et al.*, 1999). Although the GFP disperses from acrosome rapidly, other acrosomal components such as MN7 antigen and MC41 antigen remain on sperm for at least 15 min. Thus, it was shown that the acrosome reaction is not a simple all-or-none phenomenon, but one with intermediate stages. Sperm–egg interactions must be investigated taking into account factors such as an intermediate stage of acrosome-reacted sperm (Kim and Gerton, 2003).

The acrosin-GFP mice are available to the public through RIKEN BRC or CARD, Kumamoto University under the registered name, B6;C3 Tg(acro3-EGFP)01Osb or C57BL/6-Tg(CAG/Acr-EGFP)C3-N01-FJ002Osb (http://www.brc.riken.jp/lab/animal/en).

Sperm-egg interactions: an original view

PH-20 has been indicated to have a role in sperm binding with the zona pellucida, based on the finding that two out of the three monoclonal antibodies raised against PH-20 inhibited sperm–zona binding (Primakoff *et al.*, 1988). In 1993, a group studying snake venom found a significant homology of hyaluronidase to PH-20 (Gmachl and Kreil, 1993). These structural data seemed to support the long-held view that hyaluronidase plays a role in fertilization. In fact, in macaque monkeys, zona penetration was completely blocked by anti-PH-20 IgG when present during sperm–oocyte interaction (Yudin *et al.*, 1999). However, when PH-20 gene-disrupted mice were produced and examined, the mice showed a reduced ability to disperse cumulus cells but were found to be fertile (Baba *et al.*, 2002). Baba's group found a remaining hyaluronidase activity in PH-20 disrupted mouse sperm, and they discovered an additional sperm specific hyaluronidase

gene Hyal5 very close to PH-20 in the genome (Kim *et al.*, 2005). The role of hyaluronidase in sperm-egg interaction will be concluded when we see the binding ability impaired sperm from Hyal5 knockout or PH-20 and Hyal5 double knockout mice.

There are more factors suggested to be involved in sperm–zona interactions. Beta 1,4-galactosyltransferase (GalTase) is reported to function not as an enzyme, but as a sperm-egg-binding factor. Various reports exist supporting this notion. However, when a GalTase-disrupted mouse line was produced by Shur's group, unexpectedly it was found that sperm lacking GalTase could fertilize eggs and the males were not sterile although there were some minor defects in sperm-egg interactions (Asano *et al.*, 1997, Lu and Shur, 1997). This could be interpreted as suggesting that the role of GalTase was compensated by other factors and another candidate SED1 was reported as a second zona-binding factor (Ensslin and Shur, 2003). When SED1 gene was disrupted, it was found that the mice were not sterile. Such an unexpected outcome by disrupting genes for sperm-egg interaction dates back to 1994. The first gene disruption aimed at studying sperm-egg interaction was the gene for acrosin. Despite hundreds of papers supporting the importance of sperm acrosin in fertilization, acrosin-null sperm can still fertilize eggs, albeit with a slight delay compared with wild type (Adham *et al.*, 1997, Baba *et al.*, 1994). Baba's group found that protease activity persists in the sperm of acrosin gene-disrupted mouse. Altogether they found five more testis-specific proteases, from TESP1 to TESP5 (Honda *et al.*, 2002), but the roles of these enzymes in fertilization await further investigation.

There are further examples that the disruption of "important" factors results in an unexpectedly mild phenotype or shows an unpredicted phenotype in fertilization. Fertilin was originally clarified as an antigen recognized by the anti-guinea pig sperm monoclonal antibody PH-30. As the PH-30 antibody inhibited sperm from fusing with eggs, the PH-30 gene was cloned and analyzed. The antigen was found to be a heterodimer and one of the monomer genes contains a fusogenic domain similar to viral fusogen. Moreover, the other half of the heterodimer was found to contain a disintegrin domain, which binds to integrin. Thus the antigen that reacts to PH-30 was speculated to be a fusion protein and named "fertilin," which consists of fertilin alpha and beta (ADAM1/2) heterodimer(Blobel *et al.*, 1992). After this report, various types of circumstantial evidence were reported to support the role of fertilin. However, when Adam2, which forms a fertilin heterodimer, disrupted mice were produced, they found that Adam2-null sperm was shown to have fusing ability with eggs but unexpectedly, the sperm failed to bind to zona pellucida (Cho *et al.*, 1998). Later, it was found that Adam1 consisted of two independent genes: Adam1a and Adam1b (Nishimura *et al.*, 2002). Both of the ADAM1 family proteins could make a heterodimer with ADAM2, but "fertilin" on sperm surface was found to be exclusively an ADAM1b/ADAM2 type. Baba's group disrupted the ADAM1b gene but the mice were fertile without fertilin on sperm (Kim *et al.*, 2006).(This will be discussed below.) Similar stories continue in disruptions of CD46 (Inoue *et al.*, 2003), ZPBP1 (Lin *et al.*, 2007) and PKD-REJ (our unpublished data) and in some more factors (private communications).

If the fertilization mechanism consists of–redundantly prepared factors, it is not possible to analyze the role of factors with a single gene disruption experiment. If this was the case, the above mentioned factors may represent the redundant factors. If there is a factor which is not redundant and not able to be compensated, the disruption of a concerned factor must produce sterile mice. Such cases are described below.

Sperm-egg interactions: an emerging view

The first case of normally swimming sperm with normal shape and numbers failing to fertilize eggs was our report on the calmegin gene-disrupted mouse (Ikawa *et al.*, 1997). Calmegin$^{-/-}$ males are almost sterile with a lack of sperm zona-binding ability. When sperm from such males were added to cumulus-free eggs and observed under the microscope, we noted that the sperm had lost their zona-binding ability and were bouncing off the zona pellucida. However the calmegin is not directly involved

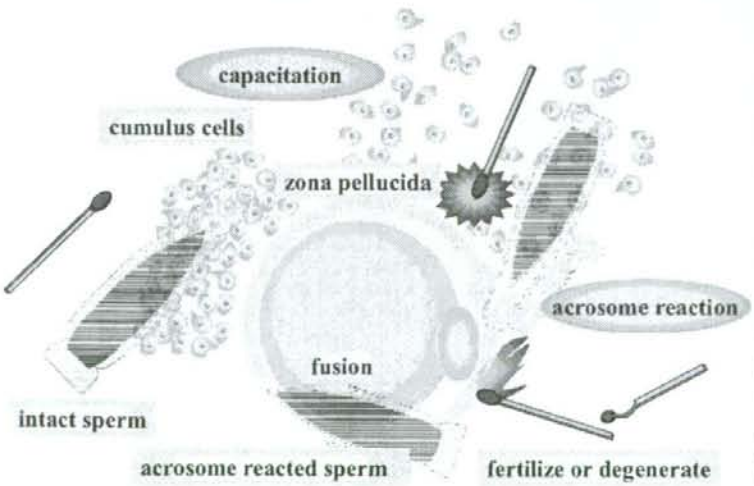

Fig. 2. Mechanism of sperm–egg interaction. *Sperm stored in the epididymis are kept metabolically inert to facilitate prolonged storage (left). Each sperm has a membranous sac over the nucleus called the acrosome which is filled with many kinds of hydrolytic enzymes. After sperm are exposed to the female reproductive environment, they become metabolically active. They undergo capacitation, which permits the acrosome reaction, and start to swim extremely vigorously (hyperactivation). Near the eggs, probably stimulated by the cumulus cells and zona pellucida, they undergo the acrosome reaction to release the acrosomal contents by exocytosis. Only acrosome-reacted sperm are known to fuse with eggs but their competency for fusion does not last long. The integrity of the acrosome in the mouse sperm can be monitored easily by using transgenic mice in which the GFP protein is targeted to the acrosomal contents (Nakanishi* et al., *1999).*

in sperm-egg interactions. Calmegin is a testis-specific homologue of the ubiquitously-expressed endoplasmic (ER) molecular chaperone calnexin. During spermatogenesis, sperm shed machineries for protein synthesis, including the ER. Thus, even in wild-type mice, there is no calmegin left on the sperm. Therefore, it is easily speculated that calmegin is acting to fold molecule(s) that are destined to function in sperm-egg binding. After we published the calmegin disruption, two other gene disruptions, Adam2 and angiotensin converting enzyme (ACE), were found to result in male sterility. Peculiarly, these knockout mouse lines and calmegin knockout mice share the phenotype of impaired zona-binding ability and, at the same time, an impaired UTJ penetrating ability. We thought the interaction of calmegin with ADAM2 was conceivable, so we immunoprecipitated calmegin from testicular lysate and examined the interaction of calmegin with ADAM2. Immunoprecipitation followed by western blot analysis revealed that ADAM1a, ADAM1b and ADAM2 formed complexes specifically with calmegin in the ER. The disruption of calmegin was shown to cause impaired heterodimerization of ADAM1a/2 and ADAM1b/2 leading to the complete loss of ADAM2 from mature sperm. Thus the reason why the calmegin and Adam2-disrupted mice share the same phenotypes became clear (Ikawa *et al.*, 2001).

To our surprise, the Adam3-disrupted male mice were also sterile with impaired zona-binding ability (Shamsadin *et al.*, 1999). Why did so many gene disruptions result in a single phenotype? This was explained by a simple reason. The amount of an ADAM family protein in sperm is easily affected by the disappearance of other ADAM family members. Baba's group found the testis specific ADAM1a was essential in fertilization, but they also found that when ADAM1a was eliminated, ADAM3 also disappeared from sperm, (Nishimura *et al.*, 2004). The synchronized disappearance of ADAM family proteins are reported by many researchers (Cho *et al.*, 1998, Ikawa *et al.*, 2001, Nishimura *et al.*, 2004, Stein *et al.*, 2005). A stunning result was reported by Baba's group. When they produced Adam1b (fertilin beta) disrupted mice, they found Adam2 also disappeared from sperm, but the sperm were fertile (Kim *et al.*, 2006). This result clearly indicates that the intensively studied protein "fertilin" is dispensable in fertilization. Combining these data together, ADAM3 emerged to be a putative key molecule in fertilization. The mechanism of infertility caused by ACE disruption remained unclear for many years. However, the relationship between ACE and ADAM3 was demonstrated. When we analyzed the ADAM3 in Ace-disrupted mice, we suggested the membrane micro-domain specific disappearance of ADAM3. We found a significant decrease of ADAM3 on Ace-/- sperm in the Triton X-114 detergent-enriched phase after phase separation, while ADAM2 remain unchanged on sperm (Yamaguchi *et al.*, 2006) (Fig. 3).

Combining these facts together, the most important factor that may participate in sperm-zona binding is tentatively ADAM3 (Yamaguchi *et al.*, 2006). However, multiple isoforms of Adam3 transcripts observed in the human were non-functional owing to the presence of deletions and in-frame termination codons (Frayne *et al.*, 1999). Therefore, ADAM3 cannot be an ultimate universal zona-binding factor among mammals.

In terms of other candidate factors for zona binding, sp56 was identified as having the characteristics expected of the sperm protein responsible for recognition of egg zona pellucida. The complementary DNA encoding sp56 was isolated and its primary sequence indicates that sp56 is a member of a superfamily of protein receptors (Bookbinder *et al.*, 1995). Zonadhesin is a multiple-domain transmembrane protein believed to function as a sperm–zona pellucida binding protein(Hardy and Garbers, 1995, Jansen *et al.*, 2001, Lea *et al.*, 2001, Wassarman, 1992). We must examine the fertilizing ability of sperm that lack these factors to reach a definite conclusion.

Moreover, it should be noted that all of the calmegin, ADAM1a-, ADAM2- and ACE-disrupted mouse sperm share the phenotype of inability, not only to bind to zona, but also to migrate into the oviduct. (Cho *et al.*, 1998, Hagaman *et al.*, 1998, Ikawa *et al.*, 2001, Nishimura *et al.*, 2004) The puzzle is why the two different inabilities of sperm–zona binding and oviduct migration run in parallel in these gene disruption experiments. We believe this phenomenon could be a good clue in helping to understand the molecular mechanisms of fertilization.

Membrane fusion

The lipid membrane has fluidity both horizontally and transversely and here are various mechanisms to maintain the lipid constitutions of both the outer and inner sides of the membrane. When the cell needs to divide, the membrane must be separated into two sections. Topologically, to achieve this, there must be a membrane break in the lipid bilayer at some point of the cytokinesis. If the egg membrane is broken artificially, as when we make a hole in the egg membrane to do intracytoplasmic sperm injection (ICSI), the opening normally seals back immediately. However, the ability for restoration is not consistent. It differs depending on the stage of the eggs. It also differs depending on the species. For example, mouse eggs are much more fragile than human eggs. We are not aware of the causes, but the adjustment and formation of a characteristic nature of the membrane must occur through a combination of various mechanisms such as the membrane undercoat and the constitution of lipids to form membrane. Sperm membrane is also known to have a complicated structure that contains cholesterol-dense "rafts" which are modified extensively during sperm capacitation (De Jonge, 2005), and evenly observed membrane by electron microscope is actually divided in some areas when examined by anti-sperm antibody.

Various important membrane fusion events exist in several tissues. For example, skeletal muscle cells are formed after myoblast cell fusion. Trophoblast cells fuse with each other to form syncytiotrophoblasts, which are necessary for implantation. Osteoclasts resorb the bone at multiple sites, occasionally containing more than 100 nuclei, and are formed by cell fusion(Yagi *et al.*, 2005). Mitochondrial membranes are known to fuse with neighboring mitochondrial membranes using mitofusin1 and mitofusion2 (Chen and Chan, 2005). One of the most precisely investigated fusion processes is exocytosis. SNAREs (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors) and some kinds of viruses appear to mimic the fusion mechanism using SNAREs for invasion. (Chan *et al.*, 1997, Lu *et al.*, 1995, Weissenhorn *et al.*, 1997). The structures of viral fusion proteins suggest that the fusion machineries employ a fundamentally similar mechanism to coalesce lipid bilayers. Fertilization is the phenomenon that involves membrane fusion between the plasma membrane of an unfertilized egg and the freshly-rear-

ranged sperm membrane soon after the acrosome reaction. Both gametes have to be conditioned properly to accomplish membrane fusion, but the factors involved in fusion were not known for many years.

Sperm–egg fusion in gene disrupted mice

Gene disruption experiments not only destroy the predicted importance of various factors, but also introduce new factors in the mechanism of fertilization. Because all who produce gene knockout mouse lines need to breed them to keep the strain alive, if there is any defect in the fertilization process, it will inevitably draw the attention of researchers in any field. For example, CD9 was disrupted aiming to determine the effects on immune functions. However, the CD9-deficient mice looked healthy and lived normally, but surprisingly, the females lacking CD9 were sterile, while the male mice remain normal (Kaji *et al.*, 2000, Le Naour *et al.*, 2000, Miyado *et al.*, 2000). We analyzed the cause of the sterility using *in vitro* fertilization (IVF) and found that the eggs had no fusing ability with sperm (Miyado *et al.*, 2000). Since fusion did not take place, the cortical granules were not released to block the polyspermy (Barros and Yanagimachi, 1971, Yanagimachi, 1994) which allowed multiple sperm to penetrate into the perivitelline space as shown in Fig. 4A. Thus, the very first discovery of an essential factor in sperm–egg fusion was found serendipitously.

CD9 is a member of the "tetraspanin" family of proteins having four transmembrane domains and ability to bind with integrins. Since integrins α6 and β1 were found on the egg membrane, the effect of the addition of synthetic integrin peptides in the IVF system was examined and the inhibition of sperm-egg fusion was reported (Chen *et al.*, 1999). However, again the gene disruption experiments in integrin α6 and β1 revealed that both integrins are not essential for eggs to fuse with sperm (Miller *et al.*, 2000).

Sperm must have completed the acrosome reaction prior to fertilization. This may imply that fusogenic factors are latent in fresh sperm and exposed only after acrosome reaction. We raised monoclonal antibodies against human sperm and screened one which did not react to fresh sperm, but to acrosome- reacted sperm. If the antibody could inhibit sperm–egg fusion, the corresponding antigen could be a good candidate as a fusion factor. Based on this hypothesis, we raised the anti-human sperm monoclonal antibody MH61 to meet

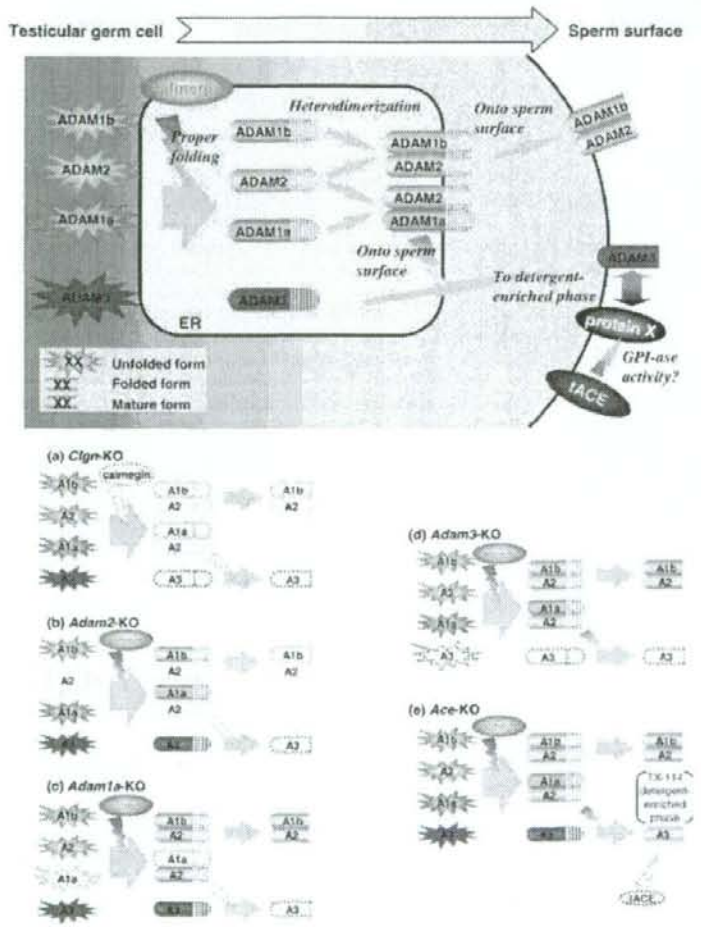

Fig. 3. Schematic model for ADAMs and their roles in sperm function. *The disruption of the genes that encode Adam1a, Adam2, and Adam3 results in impaired sperm-ZP binding. CLGN is required for the folding of ADAM1a, ADAM1b, and ADAM2 and the subsequent dimerization of these proteins. In Clgn-/-* **(a)** *and Adam2-/-* **(b)** *sperm, the disappearance of the ADAM1a/ADAM2 and ADAM1b/ADAM2 heterodimers results in the loss of ADAM1b, ADAM2, and ADAM3 from the sperm. ADAM1a is a testis-specific protein that is not found in sperm (Kim* et al., *2003). When ADAM1a is eliminated* **(c)**, *the ADAM1a/ADAM2 heterodimer disappears from the testis, whereas the expression of ADAM1b/ADAM2 is not affected. However, these sperm lack ADAM3 (Nishimura* et al., *2004). The disruption of ADAM3* **(d)** *is reported to have no significant effect on ADAM1a, ADAM1b or ADAM2 (Nishimura* et al., *2001). These findings suggest that ADAM3 is located downstream of these other ADAM proteins. The disruption of tACE leads to the aberrant localization of ADAM3* **(e)**, *most likely due to a different pathway from the one hypothesized for CLGN/ADAMs. These results indicate the importance of ADAM3 in sperm-ZP interaction and explain why disruption of the individual Ace, Clgn, Adam1a, Adam2, and Adam3 genes produces similar phenotypes.*

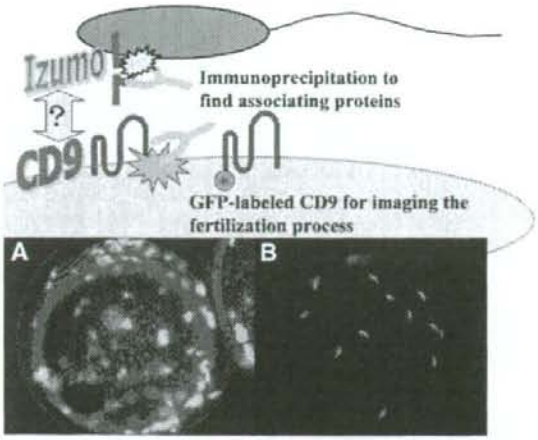

Fig. 4. Accumulation of sperm in the perivitelline space caused by failure of sperm–egg fusion. *Only one factor each on sperm and eggs is available at the moment. As in the case of live imaging acrosome reaction (ftp://ftp.gen-info.osaka-u.ac.jp/ARmovie/), the imaging of the fusion factors at the time of fertilization may be possible by producing fluorescent chimeric proteins. Immunoprecipitation will also clarify the new factors involved in the fusion event.* **(A)** *Sperm accumulated in the perivitelline space of CD9-/- mouse eggs. The sperm could penetrate the zona pellucida but failed to fuse with the egg surface. Many sperm were able to enter because of the lack of egg activation, which normally leads to cortical granule release and the zona block to polyspermy. Sperm nuclei were stained with Hoechst 33342(Miyado* et al., *2000).* **(B)** *Similarly, when eggs were inseminated with Izumo-/- sperm, the sperm could penetrate the zona pellucida but failed to fuse with the eggs. This also resulted in the accumulation of many sperm inside the perivitelline space (Inoue* et al., *2005). These penetrated sperm had clearly undergone the acrosome reaction, as they were all exposing the acrosome reacted sperm-specific antigen MN9 (Toshimori* et al., *1998).*

this criterion (Okabe *et al.*, 1990). The reacting antigen was identified as CD46, whose function is reported to be a complement receptor in human. However, when we cloned the CD46 gene in mouse, CD46 was found to be present only on sperm (Tsujimura *et al.*, 1998). We thought that this suggested the original role of CD46 was to function in sperm–egg interaction. Therefore, we produced CD46 disrupted mice with a strong expectation of causing sterility, but CD46 disrupted mice showed no visible damage to fertilizing ability in males or females (Inoue *et al.*, 2003).

We then analyzed OBF13 monoclonal antibody that was raised against mouse sperm, binds only to acrosome-reacted sperm, and inhibits sperm-egg fusion in the mouse (Okabe *et al.*, 1987). We recently identified the antigen by separation of crude extracts from mouse sperm by two-dimensional gel electrophoresis and subsequent immunoblotting with the monoclonal antibody. The identified spot was analyzed by liquid chromatography tandem mass spectrometry (LC–MS/MS). The antigen gene encodes an immunoglobulin superfamily (IgSF), type I membrane protein with an extracellular immunoglobulin domain that contains one putative glycosylation site. We termed the antigen "Izumo" after a Japanese Shinto shrine dedicated to marriage. We then produced an Izumo-disrupted mouse line. The Izumo-null males showed complete sterility despite normal mating behavior with normal vaginal plug formation. No offspring were fathered by these mice. When the sperm fertilizing ability was examined by *in vitro* fertilization system, many sperm were observed inside the perivitelline space indicating the fertilization was hampered at the sperm-egg fusion stage (Fig. 4B). However, when we performed intracytoplasmic sperm injection (ICSI) using Izumo $^{-/-}$sperm, they could activate the eggs, and the eggs were implanted normally and resulted in normal embryos. Therefore the deficiency of Izumo protein affects only to the sperm-egg fusion stage (Inoue *et al.*, 2005).

Relationship between the gene disruptions and their apparent phenotype

We need to be careful about the "off target" effects of gene disruption. A targeted disruption of the myogenic basic-helix-loop-helix gene Mrf4 is a good example. The phenotypes of three different Mrf4-disrupted mouse lines from three different laboratories with slightly different targeting vectors were very different, ranging from those showing complete viability of homozygotes to those displaying complete lethality. These three similar, but slightly different, targeting vectors had very different effects on expression of the adjacent Myf5 gene, which accounts for much of the phenotypic variation (Olson *et al.*, 1996). Another good example of the potential pitfalls of gene disruption is the case of the disruption of the Prion gene (PrP). Five independent PrP knockout mouse lines have been reported with no phenotype (Bueler *et al.*, 1992, Manson *et al.*, 1994) and with cerebellar symptoms (Moore *et al.*, 1999, Sakaguchi *et al.*, 1996, Silverman *et al.*, 2000). The discrepancy of the observation was associated with inter-gene splicing with neighboring Doppel gene in some of the targeting vectors (Flechsig *et al.*, 2003, Rossi *et al.*, 2001). To examine that the absence of Izumo directly caused the failure to fuse, we made a transgenic Izumo line driven by testis-specific calmegin promoter. The sterile phenotype was rescued with the transgenically-expressed Izumo on mouse sperm. Thus, we are certain that Izumo is the sperm factor shown to be essential for sperm-egg fusion.

Generality of sperm-egg fusion factors

In the sperm-zona binding event, ADAM3 holds the central position in the mouse, but the equivalent gene in human seems to be not producing functional protein (Frayne *et al.*, 1999). We were curious to see if Izumo is species-specific. Therefore, Izumo $^{-/-}$ sperm were mixed with hamster eggs which are able to fuse with sperm from different species. As a result, it was shown that Izumo is essential for mouse sperm to fuse with hamster eggs. Likewise, fusion of human sperm to hamster eggs was inhibited by the addition of anti-human Izumo antibody. This may suggest that Izumo is involved in sperm–egg fusion in humans as well. However, as indicated throughout this review, the addition of antibody in the IVF condition often provides us with different views obtained from various gene-manipulated animals. Therefore, it is too early to conclude that Izumo is functional in humans. It will become clear if men with mutations in their Izumo gene are found to be infertile with a symptom of fusion disability. In any case, the first

unambiguous fusion-related factors on sperm (Izumo) and on eggs (CD9) have been clarified. Again, the mice are available to the public through the Japanese animal distribution systems (indicated earlier). It is now a historic point in time, as we all are standing at the starting line of the elucidation of sperm-egg fusion.

Conclusions

Experiments using gene-manipulated animals are very powerful tools for judging the essentiality of concerned factors in fertilization. Of course, if a certain factor is judged as "not essential," it does not necessarily mean the factor is not functioning *in vivo*. However, the number of genes that are indispensable in fertilization is accumulating and their relationships with fertilization biology are emerging in the field of sperm-zona interaction. Gene disruption experiments are pursued in many research fields and the number of genes disrupted is increasing day by day. Thus, any genes that might affect reproduction will continue to be found even by researchers in different field. The day that we can draw a clear image of the sperm-egg fusion mechanism in molecular biology is definitely nearing.

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DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes

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Silencing of transposable elements occurs during fetal gametogenesis in males via de novo DNA methylation of their regulatory regions. The loss of MILI (miwi-like) and MIWI2 (mouse piwi 2), two mouse homologs of *Drosophila* Piwi, activates retrotransposon gene expression by impairing DNA methylation in the regulatory regions of the retrotransposons. However, as it is unclear whether the defective DNA methylation in the mutants is due to the impairment of de novo DNA methylation, we analyze DNA methylation and Piwi-interacting small RNA (piRNA) expression in wild-type, MILI-null, and MIWI2-null male fetal germ cells. We reveal that defective DNA methylation of the regulatory regions of the Line-1 (long interspersed nuclear elements) and IAP (intracisternal A particle) retrotransposons in the MILI-null and MIWI2-null male germ cells takes place at the level of de novo methylation. Comprehensive analysis shows that the piRNAs of fetal germ cells are distinct from those previously identified in neonatal and adult germ cells. The expression of piRNAs is reduced under MILI- and MIWI2-null conditions in fetal germ cells, although the extent of the reduction differs significantly between the two mutants. Our data strongly suggest that MILI and MIWI2 play essential roles in establishing de novo DNA methylation of retrotransposons in fetal male germ cells.

[*Keywords*: Piwi; piRNA; retrotransposon; DNA methylation; spermatogenesis]

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Argonaute proteins, also known as PAZ Piwi domain (PPD) proteins, are members of a well-conserved family that is expressed in a variety of organisms, from fission yeasts to humans. The family can be divided into two subfamilies, Piwi and Ago, based on the primary sequence homology and expression pattern of each member. Piwi subfamily members are expressed only in germ lineage cells, whereas members of the Ago subfamily are expressed ubiquitously. The PPD proteins were initially characterized as essential molecules for stem cell self-renewal and maintenance in *Drosophila*, *Caenorhabditis elegans*, and certain plant species (Cox et al. 1998; Moussian et al. 1998), and a member of the family is essential for stem cell function during regeneration in planaria (Reddien et al. 2005). There are three Piwi subfamily genes in the mouse genome: *Miwi* (*mouse piwi*), *Miwi2*, and *Mili* (*miwi-like*), which are termed *Piwil1* (piwi-like homolog 1), *Piwil4*, and *Piwil2*, respectively, in the official nomenclature. Although they are ex-

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pressed only in germ lineage cells, the expression patterns of the three genes are different during germ cell differentiation. However, it is noteworthy that both *Mili*- and *Miwi2*-targeted mice are sterile because of impaired spermatogenesis at the pachytene stage (Kuramochi-Miyagawa et al. 2004; Carmell et al. 2007).

Spermatogenesis is one of the most dramatic biological processes, as it involves cellular proliferation, differentiation, and morphogenesis. Primordial germ cells (PGCs), which emerge at embryonic day 7.5 (E7.5), reach the genital ridges up to E11.5. Male germ cells enter mitotic arrest around E13.5 and restart cell proliferation on postnatal day 2 (Sakai et al. 2004). De novo DNA methylation occurs during the quiescent phase in the fetal testes. After birth, the first wave of spermatogenesis proceeds in a synchronous manner. Following mitotic division, the first meiotic division commences on day 10. Several stages of meiosis are distinguished on the basis of differences in the constitution and relationships of the pairs of homologous chromosomes and their degrees of condensation. During differentiation, male germ cells enter the preleptotene/leptotene, zygotene, pachytene, and diplotene stages on days 10, 12, 14, and 17, respectively (Bellve et al. 1977). Thereafter, the second meiotic division takes place, with the round spermatids first appearing around postnatal day 20, and spermatogenesis continues in the adult testis. Therefore, as shown in Figure 1A, the germ cells in male gonads are different in the fetal, neonatal, and adult stages.

Recently, it has been reported that PPD proteins play key roles in many gene-silencing phenomena involving small RNAs, including microRNA (miRNA)-directed or siRNA-directed target RNA cleavage, translational repression, and chromatin silencing. Post-transcriptional gene silencing by AGO subfamily-associated miRNAs or siRNAs has been reported (Peters and Meister 2007). MILI and MIWI were recently reported to bind 26-nucleoties (nt) to 31-nt Piwi-interacting small RNAs (piRNAs), respectively (Aravin et al. 2006; Girard et al. 2006; Grivna et al. 2006; Lau et al. 2006; Watanabe et al. 2006) in the adult testis. Recently, MILI-associated piRNAs in the neonatal stage have been described as "prepachytene piRNAs" from day 10 testes (Aravin et al. 2007). However, the mechanisms of biogenesis and the biological functions of piRNAs are poorly understood compared with those of miRNAs and siRNAs.

Nearly half of the mammalian genome is composed of repeated sequences (Lander et al. 2001). Accumulating evidence suggests that PIWI-related mechanisms are involved in repressing the expression of retrotransposons, which are representative of the repeated sequences. Mutations in Piwi family genes have been shown to increase retrotransposon transcription in *Drosophila* (Sarot et al. 2004; Kalmykova et al. 2005), *Trypanosoma* (Shi et al.

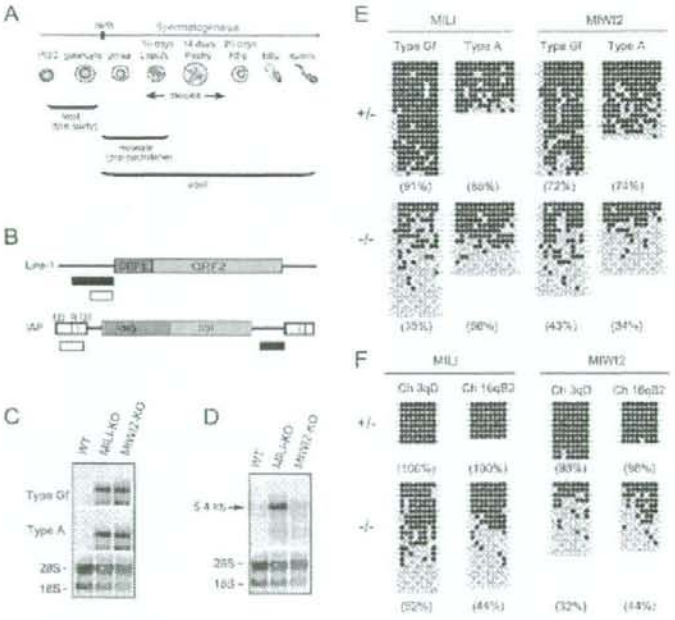

Figure 1. Expression of the IAP and Line-1 retrotransposons and methylation of their regulatory regions in neonatal prepachytene testes. (*A*) Scheme for the development of mouse male germ cells. (Gonia) Spermatogonia; (Lep) leptotene; (Zy) zygotene; (Pachy) pachytene; (RSp) round spermatid; (Esp) elongated spermatid. (*B*) Schematic diagram of the Line-1 and IAP genes. The locations of the probes used for Northern blotting and bisulfite sequencing are indicated by filled and open boxes, respectively. The sequences of the 5′-noncoding regions of the Line-1 genes are different for type Gf and type A. The probe for the 3′-noncoding region of IAP recognizes the full-length and all deletion derivatives of IAP. (*C*,*D*) Northern blotting showing transcription of the Line-1 (*C*) and IAP (*D*) retrotransposon genes in testes from 2-wk-old MILI- and MIWI2-deficient mice. The 5′-noncoding regions of type Gf and type A Line-1 and the 3′-noncoding region of IAP were used as probes. (*E*,*F*) Bisulfite sequencing of Line-1 (*E*) and IAP (*F*) in MILI- and MIWI2-deficient germ cells. Spermatogonial cells from 6- to 12-d-old mice were purified from Oct4-EGFP transgenic mouse (Yoshimizu et al. 1999) testes. (*E*) The 5′-noncoding regions of type Gf and type A Line-1 (nucleotides 874–1156; GenBank accession no. D84391) and nucleotides 1251–1542 of M13002 were analyzed. (*F*) Two LTR regions from the 5.4-kb IΔ1-type IAP in chromosomes 3qD and 16qB2 were arbitrarily chosen for analysis. Filled and open circles represent methylated and unmethylated CpGs, respectively. The percentage of methylated CpGs is shown in parentheses.