Meichroacidin Containing the Membrane Occupation and Recognition Nexus Motif Is Essential for Spermatozoa Morphogenesis*§

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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 spermatogenesis, 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, 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)XXX-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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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.
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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 (*Cliona 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 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 32P-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 (Wako, Osaka, Japan). 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 mmol/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 mM 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, 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 was performed on 35 tubules of heterozygous or homozygous mutant mice. To identify Sertoli cells, we used anti-GATA 4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). 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).
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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 × 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 significance 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. 1A), 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. 1B). MCA mRNA was not detectable using Northern blots (Fig. 1C), and the 40-kDa MCA protein was not detectable using Western blots (Fig. 1D) of the testis of homozygous MCA mutant mice. Crosses of heterozygous mutant pairs produced the expected ratios of wild-type, heterozygous, and homozygous offspring, according to classical Mendelian inheritance patterns. Homozygous mutant males had slightly smaller body masses than did wild-type males (Fig. 1E). 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. 1E), newborn pup growth rates (Fig. 1E), 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
performed. The anti-MCA polyclonal antibody stained some germ cells in testis preserved in Bouin’s solution (Fig. 2A) (2). The signal was detected in the spermatocytes and elongated spermatids (Fig. 2A), and these signals decreased gradually as morphogenesis proceeded, as reported by Tsuchida et al. (2). The heat treatment during embedding with paraffin or chemically fixed specimens might have altered the 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 that 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 testes tubes 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. S3), 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. 3D 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. 4A). Furthermore, the elongated spermatids in homozygous mutant testes were phagocytosed by Sertoli cells (Fig. 4B and supple-
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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. 5A). 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 non-ionic detergent (Triton X-100) but was mostly solubilized with potassium thiocyanate and urea (Fig. 5B). 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. 5B). 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. 5B).

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 SHIPPO1/Odf3, MCA co-precipitated with anti-Odf1 and Odf2 (Fig. 5C). Furthermore, we examined protein complexes with Septin 4 and Septin 7 (Fig. 5C) (22). No signals were detected in protein complexes with these annulus proteins. In addition,
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MCA was not detected in immunocomplexes with Hsp40 flagellum protein (6) and testis-specific centriole protein (Centrin J, Fig. 5C) (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 weakly stained. 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. 7A). 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. 7B). 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. 7B). 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
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 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.
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.

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 epididymis 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...
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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 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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