The *Mroh4* (maestro heat-like repeat family member 4) gene affects spermatogenesis and sperm morphogenesis in mice[1]

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Abstract

In our previous study, seven novel testis-specific genes were isolated from mouse pachytene spermatocyte-enriched cDNA library. Out of the genes, we here characterized the *Mroh4* (maestro heat-like repeat family member 4) gene and investigated for its function *in vivo*. First, *in silico* analysis showed that the deduced amino acid sequences of the mouse MROH4 protein contained armadillo repeat structure which mediates protein-protein interactions and had 90% identity to the rat MROH4 homologue, but no human homologue was found. Then we constructed the *Mroh4*-deficient mice by gene targeting and examined its phenotype focusing on male reproductive characteristics. The *Mroh4*-deficient mice revealed that the *Mroh4* gene influenced spermatogenesis but not fertility. The *Mroh4*$^{-/-}$ males showed about 20% reduction of mature spermatozoa in the epididymis as compared with the wild-type mice, and the spermatozoa in *Mroh4*$^{-/-}$ mice showed a significantly higher frequency of morphological aberrations of spermatozoa especially at midpiece. These results suggest that MROH4, testis-specific armadillo repeat protein plays a role in sperm production and morphogenesis of sperm flagellum by interacting with other protein(s), one of which candidates is SPAG16 that is an axonemal central apparatus protein in spermatozoa and plays a role on sperm flagellar function.

Keywords : testis-specific, armadillo repeat, *Mroh4*, gene targeting mice, abnormal spermatozoa

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1. Introduction

Spermatogenesis is a highly specialized process of cellular differentiation in which diploid germ cells of the testis differentiate into haploid spermatozoa[1]. This process is controlled by the stage-specific gene expression in the testis. After spermatozoa leave the testis, they need further maturation process to acquire fertilization competence during passing through the epididymis[2]. To clarify the molecular and cellar mechanisms that regulate mammalian spermatogenesis and spermiogenesis, a large number of novel genes have been isolated from mice by various approaches, and their functions have been investigated. Yuan *et al.*[3] isolated 42 testis-specific genes from an adult mouse testis cDNA library, and demonstrated that most of them were expressed during spermiogenesis. Fujii *et al.*[4] isolated 80 novel spermiogenesis-specific genes from the cDNA library enriched with the genes expressed during spermiogenesis, which was constructed with a stepwise subtraction technique. In our previous study,[5] we obtained a lot of EST (expressed sequence tagged) clones from a cDNA library constructed with the fraction that was enriched with mouse pachytene spermatocytes isolated using the elutriational centrifugation method. Then we performed homology search of the genes using the public database, examined their tissue-specificity of expression, and consequently found seven novel testis-specific genes. After our previous study, Choi *et al.*[6] identified 24 spermatogenic cell-specific genes from the spermatocyte UniGene library.

Over 600 mutant mice that cause reproductive defects have been produced using genetic engineering techniques[7],[8]. In those, over 400 mutants show male reproductive defects[8],[9]. Recently, the CRISPR/Cas9 system has been used to create germline mutations of testis-specific genes to investigate the molecular basis of spermatogenesis and sperm function[10]. For example, CRISPR/Cas9 mediated mouse models of the *Spata16* and *Ccdc63* genes revealed that spermiogenesis of the *Spata16* mutant was arrested at the round spermatids[11] and *Ccdc63* mutant spermatozoa has shortened flagella[12]. These model mice improve our knowledge of the genetic basis of mammalian infertility and advance clinical studies on male reproductive disorders.

Here we conducted functional analysis of a testis-specific gene, *Pac11*, which was originally isolated as a novel gene from our previous study[5], by producing its deficient mice with the gene targeting method. This gene is now named maestro heat-like repeat family member 4 (*Mroh4*). The *Mroh4* gene shows spermatocyte-specific expression and encodes a protein with armadillo (ARM) repeat[5], which mediates protein-protein interaction(s)[13]; however, its function has not been well characterized yet. The spermatogenesis of the *Mroh4*-deficient (*Mroh4*$^{-/-}$) males was phenotypically normal; however, the number of mature spermatozoa decreased. In addition, the increase of aberrations was found in flagellar morphology of the mature spermatozoa from the epididymis of *Mroh4*$^{-/-}$ mice. These observations suggest the MROH4 protein is involved in sperm production and sperm morphogenesis.

2. Materials and methods

2-1 Animals

Mice of C57BL/6J (B6) and ICR strains were purchased from Japan SLC. The animals were

kept under controlled conditions (25˚C; 14-h light and 10-h dark) and allowed intake of food and water. All experiments were performed with the consent of the Animal Care and Use Committees of Osaka University and Hokkaido University.

2-2 RNA isolation and RNA dot blot hybridization

Total RNAs were extracted from adult ovaries and testes of B6 strain mice at 4, 10, 16, 24 and 35 days after birth by the acid guanidinium-phenol-chloroform (AGPC) method[14] and purified using a mRNA Purification kit (Amersham Biosciences). 0.1 µg of denatured poly(A)$^+$ RNAs in 1 µl aliquots were spotted onto the Hybond-N+ nylon membrane (Amersham Biosciences). The membranes were baked at 80˚C for 2 h. The blots were hybridized with digoxigenin (DIG) labeled RNA probes, washed, and detected as previously described[5].

2-3 *In silico* analysis

The full-length cDNA sequence and the deduced amino acid sequence of the mouse *Mroh4* gene were taken from the FANTOM (Functional Annotation of Mouse) database of RIKEN. The cDNA sequence was used to search genome DNA sequence of the mouse *Mroh4* gene by Celera Discovery System™ (http://www.celera.com/), and then these sequences were compared with each other to confirm exon-intron structure. The possible protein structure was estimated by the SUPERFAMILY database (http://supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY/). Mammalian homologues of the mouse *Mroh4* gene were searched using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The deduced amino acid sequences of the mouse and rat MROH4 proteins were aligned using the CLUSTAL W multiple sequence alignment program (http://www.ebi.ac.uk/clustalw/index.html).

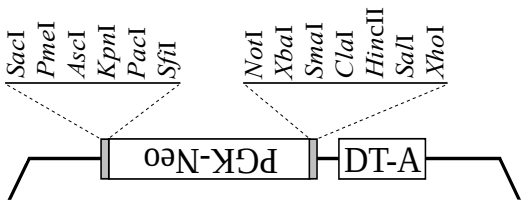

Fig. 1 Map of pMulti-ND-1.0 targeting vector. The unique restriction enzyme sites are inserted at the 5' side of phosphoglycerine kinase-neomycin (PGK-Neo) cassette. The region with 3' multi restriction sites, which is included in original pBluescript II KS (+) vector, is located between PGK-Neo cassette and diphteria toxin A-fragment (DT-A) cassette.

2-4 Construction of pMulti-ND-1.0 targeting vector

A 1.6 kb blunted *Not*I-*Xho*I fragment isolated from pMC1DTpA[15], which contains the diphteria toxin A-fragment (DT-A), was inserted into blunted *Kpn*I-*Xho*I sites of pBluescript II KS (+) vector (pBS) (Stratagene), resulting in pBS-DT. A 1.7 kb *Not*I fragment without *Xho*I site isolated from pTransCX-*GFP*:*Neo*[16], which contains phosphoglycerine kinase-neomycin (PGK-Neo) cassette flanked by two loxP sites, was inserted in a reverse direction toward DT-A into the *Not*I site of pBS-DT, resulting in pBS-DT-Neo. Linker oligonucleotide which contained

*Sac*I-*Pme*I-*Asc*I-*Kpn*I-*Pac*I-*Sfi*I sites in this order was inserted into the *Sac*I-*Sfi*I sites of pBS-DT-Neo (Fig. 1), resulting in pMulti-ND-1.0 targeting vector (pMultiND1). The other unique sites of *Not*I-*Xba*I-*Sma*I-*Cla*I-*Hin*cII-*Sal*I-*Xho*I in this order were also located between PGK-Neo and DT-A in pMultiND1 (Fig. 1).

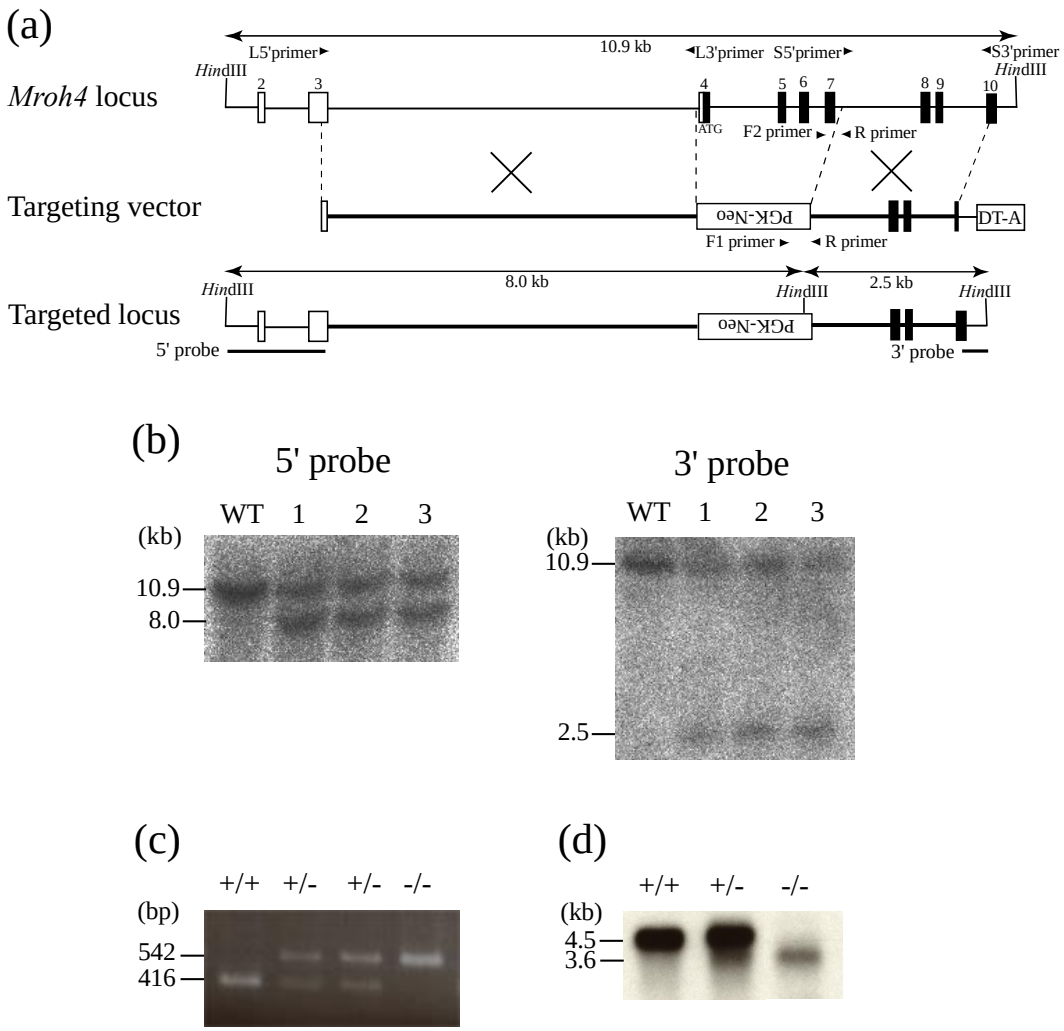

Fig. 2 Targeting of the *Mroh4* locus by homologous recombination. (a) Graphic representation of the genomic *Mroh4* locus, the targeting vector, and the targeted locus. Exons and introns are represented by vertical boxes and horizontal lines, respectively. Open box and closed box indicate untranslated region and coding region, respectively. (b) Southern blot analysis of three targeted ES cell clones. The wild-type allele presented an 10.9 kb *Hin*dIII band, while the disrupted alleles were represented as a 8.0 kb band with a 5' hybridization probe and a 2.5 kb band with 3' probe. (c) Genotyping by PCR analysis. Tail genomic DNA was amplified with primers specific for the wild-type (primers F2 and R; 416 bp) and disrupted (primers F1 and R; 542 bp) alleles. (d) Northern blot analysis of poly $(A)^+$ RNAs (1 μg) isolated from testes. The wild-type band disappeared, but in the testis of *Mroh4*$^{-/-}$ mice the lower molecular band was weakly observed. *Mroh4*$^{+/-}$ testes contained both the wild-type band and the weak band of the deficient allele.

2-5 Construction of the *Mroh4* gene disruption vector

The targeting construct contained a 5.2 kb noncoding sequence in the upstream of the ATG initiation codon partially encompassing exon 3 extending to near exon 4 of the *Mroh4* gene in the 5' homology arm and a 2.0 kb genomic fragment ranging from intron 7 to part of exon 10 of the *Mroh4* gene in the 3' homology arm. These 5' and 3' arms were amplified by Expand High Fidelity PCR System (Roche Diagnostics) using whole genomic DNA of the R1-S3 ES cell clone[17] as a template. The PCR primers used were as follows: 5'-GCGGCGCGCCAAGGGATGGAAAGGTGACAACGAAGGATCG-3' (L5' primer, 5' primer of the 5' arm with *Asc*I site underlined is contained), 5'-GCTTAATTAATTCCAGAAGCAGAGGTGGGCAATACAAGCC-3' (L3' primer, 3' primer of the 5' arm with *Pac*I site underlined is contained), 5'-GGAGCGGCCGCCCATTGGTGACACTAGGGCTCTTGCCTGG-3' (S5' primer, 5' primer of the 3' arm with *Not*I site underlined is contained), and 5'-GCGAGTCGACCTGGGACTTGAGAAGGGGTTTGAATCTGGG-3' (S3' primer, 3' primer of the 3' arm with *Sal*I site underlined is contained) (Fig. 2a). A 5.2 kb 5' arm and a 2.0 kb 3' arm were inserted into a 6.1 kb pMultiND1 at the sites of *Asc*I and *Pac*I and at the sites of *Not*I and *Sal*I, respectively (Fig. 1); the 13.3 kb targeting vector was finally constructed. Prior to its electroporation into ES cells, the targeting vector was linearized with *Pme*I site. If homologous recombination occurs successfully between the targeting construct and the endogenous *Mroh4* allele, exons 4 to 7 of the *Mroh4* gene is deleted.

2-6 Generation of *Mroh4*$^{-/-}$ mice

1×10^7 R1-S3 ES cells were electroporated with 25 μg of the linearized targeting construct (0.4 cm cuvette, 240V, 500 μF) and then plated onto feeder cells. This trial was performed three times. After 7-day selection with 150 μg/ml of G418, resistant colonies were harvested. Each colony was divided for culture and PCR screening. Positive clones were expanded, and genomic DNA was prepared from them, and the occurrence of recombination was confirmed by Southern blot hybridization.

To produce *Mroh4*-deficient mice, three lines of monoallelically targeted ES clones with normal karyotypes were injected into (B6 × DBA2) F_1 blastocysts at 3.5 dpc. The blastocysts were transferred to pseudopregnant foster mothers of the ICR strain, and consequently the chimeric mice derived from three targeted ES cell lines were produced. Only male chimeric mice with partial agouti coat color were mated with B6 females. The F_1 agouti offspring were then subjected to genotyping. The *Mroh4*$^{-/-}$ mice were produced by the intercrossing of heterozygous F_1 offspring mice. The mouse line with B6 genetic background was established by mating F_2 offspring (B6/129 genetic background) with B6 mice and then further backcrossing to B6 mice for five generations. These mutant mice are available from RIKEN BioResource Center (http://www.brc.riken.jp/) as BRC no. 01599 for B6 background.

2-7 Southern blot hybridization and PCR analysis

For Southern blot hybridization, genomic DNA of the targeted ES cells was extracted. *Hin*dIII-digested genomic DNA (approximately 15 μg) was fractionated in a 1% agarose gel, transferred to a Hybond-N+ membrane (Amersham Biosciences), and hybridized with the ^{32}P-labeled probe. The genomic DNA sequences of the *Mroh4* gene between exon 1 and intron 3 and between intron 9 and exon 11 were used as 5' and 3' probes to find targeted clones (Fig. 2a).

For genotyping, genomic DNA was prepared from tails and used as the template for PCR analysis. PCR was performed using two forward primers, and one reverse primer (R primer, 5'-CAAAGTAGACCTTTCATCCATCCCGTGTCC-3') that is specific for the sequence in both the wild-type and targeted allele. One of two forward primers is specific for the PGK promoter that is contained in the targeted allele (F1 primer, 5'-TAGTGAGACGTGCTACTTCCATTTGTCACG-3'), and the other forward primer (F2 primer, 5'-ACTGCATTGGACACTTCACCAGCTTTCCAC-3') for the sequence that is present in the wild-type allele of intron 6, which is disrupted in the targeted allele. F1 and R primer pair yielded a 542 bp fragment of the mutant allele. F2 and R primer pair yielded a 416 bp fragment of the wild-type allele. This PCR genotyping allowed to accurately discriminate mice with the wild-type allele, and mice heterozygous and homozygous for the targeted allele.

2-8 Northern blot hybridization

Northern blot hybridization was performed as described previously[5]. Briefly, total RNAs were extracted from testes of 8-week-old *Mroh4*$^{-/-}$, *Mroh4*$^{+/-}$ and *Mroh4*$^{+/+}$ mice by the AGPC method[14], and purified using a mRNA Purification kit (Amersham Biosciences). One μg of poly(A)$^+$ RNAs were subjected to electrophoresis with 1% gel and transferred to the Hybond-N+ nylon membrane (Amersham Biosciences). The *Mroh4* RNA probe was prepared by *in vitro* transcription of a 2692 bp cDNA fragment using a DIG RNA Labeling kit (Roche Diagnostics) and hybridized with the membrane at 68ºC for 2 h in DIG Easy Hyb solution (Roche Diagnostics). The membrane was washed in 0.1% SDS/1× SSC for 15 min twice and 0.1% SDS/0.05× SSC at 68°C for 1 h twice. The luminescent signals were generated with anti-DIG-AP Fab fragments and CDP-Star (Roche Diagnostics), and detected by exposing the membrane to BioMax MS-1 Autoradiography Film (Carestream Health, USA).

2-9 Histological analysis

Testes of *Mroh4*$^{-/-}$ and *Mroh4*$^{+/+}$ mice in B6 background were fixed overnight in Bouin's solution, and stored in 70% ethanol at room temperature until use. The tissue sections were prepared using a standard protocol; the testes were dehydrated by graded washes with ethanol and xylene, embedded in paraffin wax, and cut into sections of 5 μm thickness. The sections were mounted onto glass slides, treated in a series of graded xylenes and ethanols to dissolve paraffin, and stained with Carazzi's hematoxylin and eosin for histopathological evaluation.

2-10 Assessment of fertility and fecundity

To assess fertility and fecundity of *Mroh4*-deficient mice with B6 background, one male (≥12 weeks old) mouse of *Mroh4*$^{-/-}$ or *Mroh4*$^{+/+}$ genotype was mated with two wild-type B6 female (≥8 weeks old) mice for 2 months. The number of mice achieving pregnancy and the number of offspring obtained from each mating set were recorded.

2-11 Epididymal sperm counts

Two cauda epididymides were taken from each *Mroh4*$^{-/-}$ and *Mroh4*$^{+/+}$ mice with B6 background, minced in 1 ml of PBS for each tissue and incubated at 37˚C for 30 min. After incubation, the spermatozoa were taken out from tissue pieces by pipetting, and then the suspension was passed through Cell Strainer with an 40-μm-pore-size nylon filter (BD Biosciences). And then total number of spermatozoa in two cauda epididymides was calculated using a hemocytometer.

2-12 Epididymal sperm morphology

Spermatozoa from one fourth of cauda epididymides of *Mroh4*$^{-/-}$ and *Mroh4*$^{+/+}$ mice in B6 background were dispersed in PBS at 37˚C for 30 min and stained with 0.05% Eosin Y (Wako Pure Chemical Industries) for 20 min at room temperature. Two droplets of the sperm suspension were smeared on the slides and air-dried. The slides were examined under light microscopy at a magnification of ×400. Sperm morphology was examined for 200 spermatozoa per male.

2-13 Statistical analysis

For assessment of fertility, Student's *t*-test was used to approve the differences of the mean value in the litter size. The differences of the mean value in the weight of body, testis, seminal vesicle, and epididymis among the three genotypes (*Mroh4*$^{-/-}$, *Mroh4*$^{+/-}$ and *Mroh4*$^{+/+}$) were approved by one-way analysis of variance (one-way ANOVA). Post Hoc test was used to approve the differences of the mean value in the weight of cauda epididymides and the number of epididymal spermatozoa. Mann-Whitney test was used to approve the differences of the mean value in the frequency of abnormal spermatozoa. Values are given as mean ± standard deviation (SD). These statistical analyses were conducted by the statistical program SPSS Base 10.0 (SPSS Inc., USA).

3. Results

3-1 Expression of mouse *Mroh4* mRNA

Northern blot analysis demonstrated a 4.5 kb transcript of the *Mroh4* gene in the mouse testis but did not detect mRNA in the brain, heart, kidney, liver, lung, skeletal muscle, and spleen as previously reported[5]. No transcripts were observed in the ovary by RNA dot blot analysis (Fig. 3). These data reveal that the mouse *Mroh4* gene is only involved in male gametogenesis. During the development of male germ cells, the transcript was not detected in neonatal mouse testis until 16 days of age (Fig. 3), indicating that the expression of mouse *Mroh4* mRNA is supposed to start around pachytene stage because pachytene spermatocytes first appear around 14 days after birth[18]. This is consistent

with our previous data that mouse *Mroh4* mRNA was located in spermatocytes by *in situ* hybridization analysis[5]. Our results totally coincide with transcript analysis of *Mroh4* gene using RT-PCR by Choi *et al*.[6]

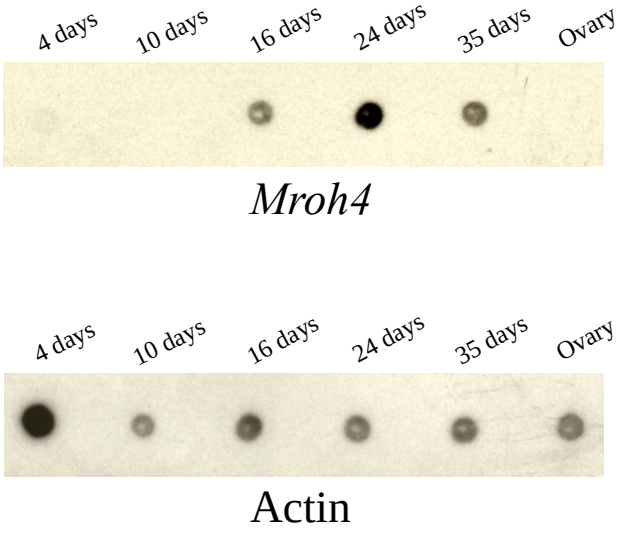

Fig. 3 Dot blot hybridization of mouse *Mroh4* mRNA in various postnatal developmental stages of testes and adult ovary. 0.1 μg of poly(A)$^+$ RNA extracted from prepubertal mouse testes and 8-week-old mouse ovary was used. The blots were probed with mouse *Mroh4* and actin RNA probes. The signals of *Mroh4* were detected from 16 days of age in testes, but no signals were observed in ovary. Tunicae were not removed for the testes of 4-days-old mice.

3-2 Genomic organization of the mouse *Mroh4* gene and its deduced amino acid sequence

The genomic organization of the mouse *Mroh4* gene was examined by comparison of the full-length cDNA sequence taken from the FANTOM database with the genomic sequence in the database of Celera Genomics. This comparison revealed that the mouse *Mroh4* gene contains 27 exons and spans roughly 30 kb on the mouse chromosome 15. The ATG-starting codon is located on exon 4, and the stop codon on the final exon 27. The cDNA sequence without a poly(A) tail contained 3493 nucleotides, and its open reading frame (ORF) encoded 983 amino acid residues (Fig. 4). The complete nucleotide sequence of the *Mroh4* gene and its deduced amino acid sequence are available from the DDBJ/EMBL/GenBank Data Bank (accession no. AK006048 and BAB24382, respectively). The mouse MROH4 amino acid sequence had predicted armadillo (ARM) repeat regions (Fig. 4) as deduced via the SUPERFAMILY database. The *E* value for the ARM repeat was 1.1e-14, suggesting that the mouse MROH4 protein contains the ARM repeat structure.

3-3 Homologues of the mouse *Mroh4* gene in other mammals

The deduced amino acid sequence of the mouse MROH4 protein showed 90% amino acid identity to the rat MROH4 protein (accession no. XP_235415) (Fig. 4). The rat MROH4 protein showed 91% amino acid identity with the mouse MROH4 protein in the ARM repeat region. The rat

MROH4 amino acid sequence also showed predicted ARM repeat regions (Fig. 4). However, database search using blastn and blastp showed no obvious human homologues.

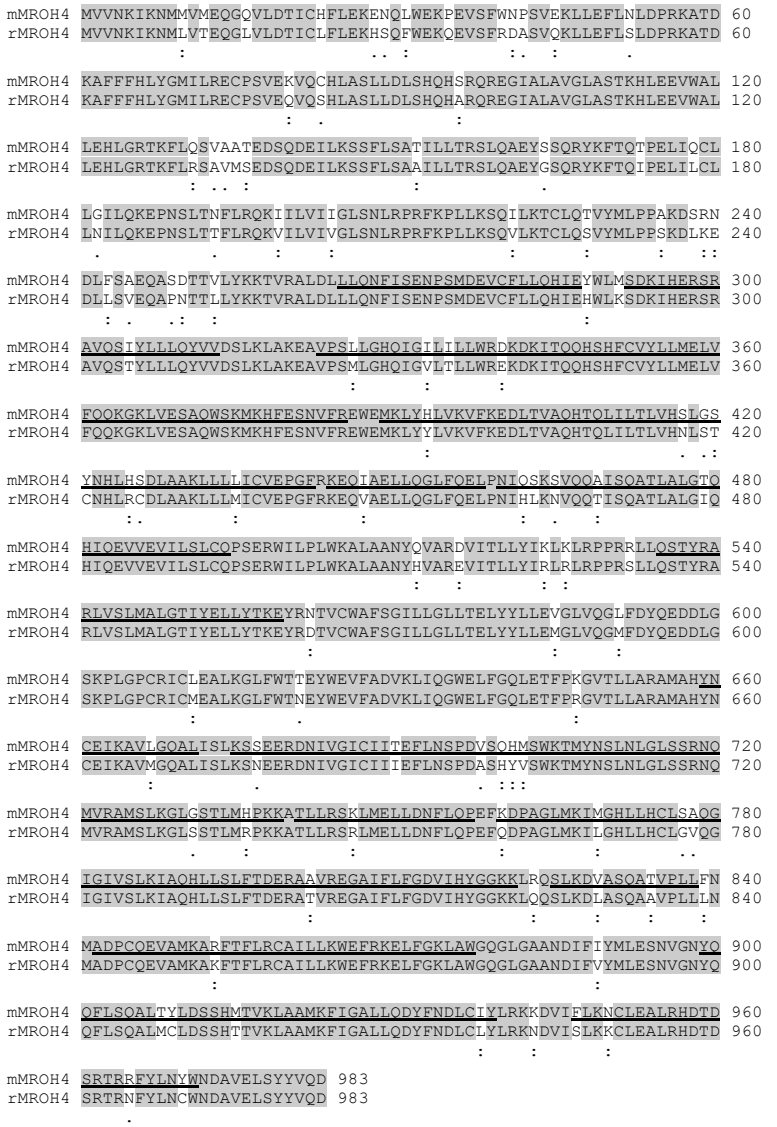

Fig. 4 Multiple alignment of the amino acid sequences of the mouse and rat MROH4 (mMROH4; rMROH4, respectively) proteins by the CLUSTAL W multiple sequence alignment program. Identical residues are shown as shaded gray. (:) and (.) indicate positions which have similar residues with a score more than 0.5 and a score less than 0.5, respectively. Underline indicates the predicted armadillo repeat region.

3-4 Targeted disruption of the *Mroh4* gene using pMulti-ND-1.0 targeting vector

Five of 432 G418-resistant ES clones were screened by PCR for checking the occurrence of homologous recombination (data not shown). One of the five clones did not grow well in the cell

culture, and one of the other four clones showed abnormal karyotype. The remaining three clones, which had normal karyotypes, were subjected to Southern blot hybridization to detect the recombination on the 5' and 3' ends of the *Mroh4* locus (Fig. 2b). The wild-type allele corresponded to an 10.9 kb *Hin*dIII band, while the disrupted alleles were detected as a 8.0 kb band with 5' probe and a 2.5 kb band with 3' probe.

3-5 Establishment of *Mroh4*$^{-/-}$ mice

The results of PCR genotyping demonstrated that crosses of heterozygous mice yielded offspring with three possible genotypes (Fig. 2c) that were almost segregated at the Mendelian ratio (*Mroh4*$^{+/+}$; n = 179 (26.6%), *Mroh4*$^{+/-}$; n = 344 (51.1%), and *Mroh4*$^{-/-}$; n = 150 (22.3%)). Northern blot hybridization detected a mRNA band that is smaller than 3.6 kb in *Mroh4*-deficient mice; however, its intensity was very weak (Fig. 2d). *Mroh4*$^{-/-}$ male and female mice were normal in health condition, body weight and organ sizes, and behavior. The similar results were obtained in *Mroh4*$^{-/-}$ males and females in the other two mouse lines that were derived independently from different ES clones.

3-6 Reproductive organ weight of *Mroh4*$^{-/-}$ mice

Weight of reproductive organs was examined for 10 mice of each *Mroh4*$^{-/-}$, *Mroh4*$^{+/-}$ and *Mroh4*$^{+/+}$ genotypes with B6 background at 24 weeks of age. The testes weight was significantly different between *Mroh4*$^{-/-}$ and *Mroh4*$^{+/+}$ mice; the testes weight was 193.3 $\pm$ 18.4 mg on average for *Mroh4*$^{-/-}$ males, and 204.9 $\pm$ 16.0 mg for *Mroh4*$^{+/+}$ males ($P < 0.05$). Meanwhile, weight of seminal vesicles and epididymides was not significantly different among the three genotypes (Table 1).

Table 1 Weight of reproductive organs in *Mroh4*-deficient males at 24 weeks of age (n = 10)

Mroh4	Weight of reproductive organs		
genotype	Testis (mg)	Seminal vesicle (mg)	Epididymis (mg)
+/+	204.9 ± 16.0	299.4 ± 34.8	68.2 ± 5.8
+/-	214.6 ± 8.7	302.5 ± 46.2	69.9 ± 3.6
-/-	193.3 ± 18.4*	308.6 ± 35.5	66.0 ± 5.0

*Significantly different from the result of *Mroh4*$^{+/+}$ mice ($P < 0.05$).

3-7 Spermatogenesis in *Mroh4*$^{-/-}$ mice

To estimate the function of the *Mroh4* gene acculately, we examined the condition of spermatogenesis in 8-week-old males of the congenic line of *Mroh4*-deficient mice with B6 background, which was established by backcross mating of *Mroh4*$^{+/-}$ males with B6 females for five generations. Light microscopy of testis sections of *Mroh4*$^{-/-}$ mice showed normal structure of the seminiferous tubules and interstitium (data not shown). A large number of spermatozoa were also

observed in the *Mroh4*$^{-/-}$ testes. Spermatogenesis was normal in *Mroh4*$^{+/+}$ males, and a large number of spermatozoa were also observed. These results indicated that spermatogenesis seems to be normal in *Mroh4*$^{-/-}$ mice with B6 background; however, the difference of testis weight suggests that the *Mroh4* gene may be involved in spermatogenesis.

3-8 Fertility of *Mroh4*$^{-/-}$ mice

Fertility of *Mroh4*$^{-/-}$ males with B6 background was studied by mating *Mroh4* mutant males with mature female mice of B6 strain for two months. Ten *Mroh4*$^{-/-}$ males produced 41 litters, and all of them were fertile. The average litter size obtained from *Mroh4*$^{-/-}$ males (8.1 $\pm$ 1.5) was not significantly altered as compared with *Mroh4*$^{+/+}$ males (7.8 $\pm$ 1.9) ($P > 0.05$). In conclusion, *Mroh4*$^{-/-}$ males showed normal fertility.

3-9 Number of mature spermatozoa in the epididymides of *Mroh4*$^{-/-}$ mice

The sizes of cauda epididymides of 24-week-old mice were not significantly different between *Mroh4*$^{-/-}$ and *Mroh4*$^{+/+}$ males. Spermatozoa in the epididymides of six *Mroh4*$^{-/-}$ and six *Mroh4*$^{+/+}$ mice were counted, and the results indicated that *Mroh4*$^{-/-}$ mice showed about 20% reduction of spermatozoa significantly ($P < 0.05$) compared to *Mroh4*$^{+/+}$ mice (Table 2).

Table 2 Numbers of spermatozoa in the cauda epididymides of *Mroh4*-deficient males at 24 weeks of age (n = 6)†

Mroh4 genotype	Cauda epididymis weight (mg)	No. of epididymal spermatozoa ($\times 10^6$)
+/+	23.9 ± 1.0	29.2 ± 1.7
-/-	22.0 ± 1.8	24.0 ± 2.1*

†Spermatozoa were collected from two epididymides for each male.
*Significantly different ($P < 0.05$).

3-10 Sperm morphology in the epididymides of *Mroh4*$^{-/-}$ mice

Mature spermatozoa were collected from the cauda epididymides of five of each 24-week-old *Mroh4*$^{-/-}$ and *Mroh4*$^{+/+}$ mice. Light microscopic observation showed that 24.5% of spermatozoa were abnormal in *Mroh4*$^{-/-}$ mice. Abnormalities were mainly observed in the midpiece of flagellum as shown in Fig. 5b. The midpiece was 180˚ bent over the principal piece in most abnormal spermatozoa (Fig. 5b upper). The other spermatozoa showed a folded flagellum at the proximal midpiece (Fig. 5b middle) or flagellar angulation at the distal midpiece (Fig. 5b bottom). The frequency of abnormal spermatozoa whose flagella were bent at midpiece was 3.1% in *Mroh4*$^{+/+}$ mice, and this frequency was significantly ($P < 0.01$) increased to 12.6% in *Mroh4*$^{-/-}$ mice (Table 3).

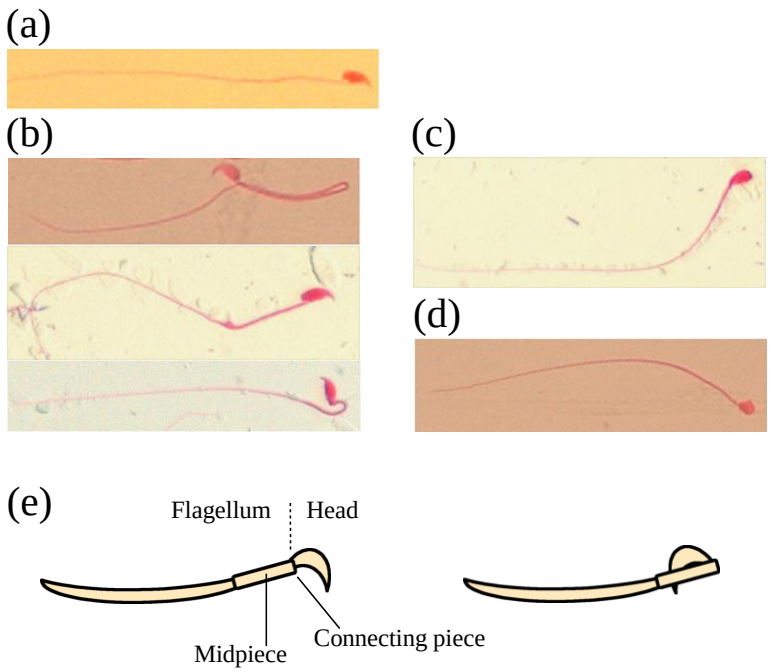

Fig. 5 Examples of abnormally shaped spermatozoa in the epididymis of *Mroh4*-deficient males. (a) Normal spermatozoon, (b) Midpiece abnormality: spermatozoa with flagella folded at the midpiece, (c) Connecting piece abnormality: spermatozoon with 180˚ bent back head at the connecting piece between head and flagellum, and (d) Head abnormality: spermatozoon with abnormally shaped head. (e) Schematic diagrams of normal spermatozoon (left) and spermatozoon with connecting piece abnormality (right).

Table 3 Frequencies of abnormal spermatozoa in the epididymides of *Mroh4*-deficient males at 24 weeks of age (n = 5)†

Mroh4			Abnormal (%)	
genotype	Normal (%)	Midpiece	Connecting piece	Head
+/+	87.9	3.1	4.2	4.8
-/-	75.5**	12.6**	5.2	6.7

†Two hundred spermatozoa were observed per male.

**Significantly different ($P < 0.01$).

4. Discussion

First, we investigated the evolutionary conservation and divergence of sequence properties of the testis-specific *Mroh4* gene. There was 90% amino acid identity between the rat and mouse MORH4 proteins, suggesting that the rat homologue has the same function as the mouse MORH4 protein. However, no human homologues were found from current public databases of the human genome

sequences, although the pseudogene, *Mroh4p*, is existed. Comparison of amino acid sequences between closely related species has shown that genes involved in reproduction, which mediate spermatogenesis and fertilization, have a tendency to diverge rapidly. One of the most rapidly evolved gene was the transition protein 2 (*Tnp2*) gene, and its amino acid sequence divergence was 68% between mouse and human[19]. Also in the *Morh4* gene, rapid base substitution might have occurred between its rodent and human homologues during the process of evolution.

To clarify the function of this gene, we then produced the *Mroh4*-deficient mouse by the gene targeting method. For construction of the *Mroh4* gene disruption vector, we first developed the pMulti-ND-1.0 targeting vector to perform the gene targeting efficiently. This vector contains the Neo-resistance gene (*neo*^{r}) as a positive selection marker and DT-A as a negative selection marker, and some rare restriction enzyme sites that are located at the 5' side of *neo*^{r} and between *neo*^{r} and DT-A. We amplified the long and short arm fragments with restriction enzyme sites that are located between L5' primer and L3' primer and between S5' primer and S3' primer, respectively (Fig. 2a) using the PCR method and introduced them to pMulti-ND-1.0 targeting vector. To find the replication errors of PCR, we checked the sequence of the short arm fragment using the database of Celera Genomics; however, the sequencing for the long arm fragment was not performed. Five of 432 (1.2%) G418-resistant ES clones underwent homologous recombination that disrupted the *Mroh4* gene. This system was also used for producing the knockout mouse of the *Izumo1* gene, and the similar efficiency of correct homologous recombination was obtained (1.0%, 4/385 clones)[20]. This targeting efficiency is comparable to those obtained by the usual gene targeting method using the vector that was constructed with the genomic DNA fragments isolated from the genomic DNA library, suggesting that our method using PCR-amplified 5' and 3' arms economize time required for gene targeting of any genes.

International Mouse Phenotyping Consortium generated and characterized as many as 5000 knockouts[21], in which the *Mroh4*-deficient mouse is included. The *Mroh4*-deficient mouse produced in this study did not show any distinctive morphological phenotypes such as abnormalities in integument and hear growth as shown in Mouse Genome Informatics (http://www.informatics.jax.org/). Therefore, we then examined phenotypes associated with spermatogenesis and fertility in the *Mroh4*-deficient mouse.

Histological analysis of testes showed normal spermatogenesis in *Mroh4*$^{-/-}$ mice; however the *Mroh4*$^{-/-}$ testis was significantly smaller than that of *Mroh4*$^{+/+}$ mice ($P < 0.05$), indicating that the *Mroh4* gene has some role in spermatogenesis. Actually, the number of mature spermatozoa decreased significantly ($P < 0.05$) in the epididymis of *Mroh4*$^{-/-}$ males. Morphological abnormality of spermatozoa was also significantly higher, especially at midpiece than *Mroh4*$^{+/+}$ males; however, *Mroh4*$^{-/-}$ male mice have normal fertility. These phenotypes of *Mroh4*$^{-/-}$ mice are similar to those of male mutant mice that lack the TAF7L. The *Taf7l* gene is not involved in fertility but affects the number and morphology of spermatozoa[22]. TAF7L is a testis-specific form of the generally expressed TAF7 (TATA-binding protein-associated factor 7) which is important for the transcription in spermatogenesis. TAF7L protein is localized to cytoplasm in spermatogonia and early

spermatocytes, and is transferred into the nuclei of pachytene spermatocytes and round spermatids[23]. The number of sperm in *Taf7l*-deficient males were reduced by about 60%. The flagella were folded or angulated at the midpiece and/or the junction between the midpiece and principal piece in around 50 % of *Taf7l*$^{-/Y}$ spermatozoa[22].

Subcellular localization of MROH4 protein in the Goldi apparatus was shown by the transfection assay using GC-2 cells[6]. Acrosome, which is derived from Goldi apparatus during spermiogenesis, is the organelle on sperm head; therefore, MROH4 may be involved in a the function of acrosome. ARM repeat proteins interact with many other proteins and play important roles in a great diversity of cellular processes[13],[24]. Identification of partner protein that interacts with mouse MROH4 should provide a clue to elucidate the role of the MROH4 protein in sperm maturation. *Spag16* gene encodes an axonemal central apparatus protein in spermatozoa and plays a role in sperm flagellar function[25],[26]; moreover, a yeast-two-hybrid system identified MROH4 to be a potential protein that associates with SPAG16[27]. This observation provides a clue to understand the factor causing the morphological aberration of sperm midpiece in *Mroh4*$^{-/-}$ mice. The *Mroh4*$^{-/-}$ mouse in the present study revealed that the *Mroh4* gene influenced spermatogenesis (Table 1 Weight of testis) but not fertility. Therefore, we suggest that testis-specific MROH4 containing the ARM repeat affects sperm number (Table 2) and morphogenesis (Table 3) especially at midpiece in mice. This study provides a fundamental information about the sperm production and morphogenesis, which may help to understand the human male disease of sperm abnormality.

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