A Mutation in the Nuclear Pore Complex Gene Tmem48 Causes Gametogenesis Defects in Skeletal Fusions with Sterility (sks) Mice*

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Background: sks is a mouse mutant showing sterility caused by defects in meiosis.

Results: We found a mutation of the Tmem48 gene encoding nuclear pore complex protein. The mutation causes aberrant splicing, resulting in deletion of an exon.

Conclusion: Tmem48 is essential for meiosis and gametogenesis.

Significance: This is the first report to demonstrate that the nuclear pore complex has an important role in mammalian gametogenesis.

Skeletal fusions with sterility (sks) is an autosomal recessive mutation of mouse that results in male and female sterility because of defects in gametogenesis. The mutants also have skeletal malformations with fused vertebrae and ribs. We examined testicular phenotypes of sks/sks mice to investigate the defects in spermatogenesis. Histological and immunocytochemical analyses and expression analyses of the marker genes demonstrated that spermatogenesis is arrested at mid to late pachytene stage of meiotic prophase with defective synopsis of the homologous chromosomes. Next, we determined the precise chromosomal localization of the sks locus on a 0.3-Mb region of mouse chromosome 4 by linkage analysis. By sequencing the positional candidate genes in this region and whole exome sequencing, we found a GG to TT nucleotide substitution in exon 6 of the Tmem48 gene that encodes a putative transmembrane protein with six transmembrane domains. The nucleotide substitution causes aberrant splicing, which deletes exon 6 of the Tmem48 transcript. Specific expression of TMEM48 was observed in germ cells of males and females. Furthermore, the phenotypes of the sks mutant were completely rescued by the transgenesis of a genomic fragment containing the wild-type Tmem48 gene. These findings indicate that the Tmem48 mutation is responsible for the gametogenesis defects and skeletal malformations in the sks mice. The TMEM48 protein is a nuclear membrane protein comprising the nuclear pore complex; its exact function in the nuclear pore complex is still unknown. Our finding suggested that the nuclear pore complex plays an important role in mammalian gametogenesis and skeletal development.

Mammalian gametogenesis, particularly spermatogenesis, is a dramatic developmental process. It consists of cell proliferation, differentiation, and morphogenesis, which involve numerous cellular and molecular steps, including germ cell differentiation and germ/somatic cell interactions. In mouse, spermatogenesis begins 7 days after birth with the differentiation of gonocytes into spermatogonial stem cells and subsequent recruitment of differentiated spermatogonia into meiosis I with the formation of pre-leptotene primary spermatocytes. Meiosis I is characterized by a prolonged prophase, consisting of the leptotene, zygotene, pachytene, diplotene, and diakinesis stages, in which the pairing and recombination of homologous chromosomes are accompanied by changes in chromatin structure. Meiosis I is initiated by the formation of meiotic DNA double-strand breaks (DSBs) at the leptotene stage and these DSBs are repaired by homologous recombination in zygote spermatocytes. During the zygote and pachytene stages, homologous chromosomes undergo synopsis, a process mediated by the formation of the synaptonemal complex (SC), which plays an essential role in meiotic recombination (1). At the diplotene.diakinesis stages, the synaptonemal complexes disintegrate; following this, the condensed bivalent chromosomes align on the metaphase plate, and sister chromatids dissociate into two daughter cells.

To study the molecular mechanisms underlying mammalian gametogenesis, mutant mice with defective gametogenesis are useful animal models to identify the key molecules and signaling pathways (1, 2). So far, a large number of infertile and subinfertile animal models have been obtained by spontaneous mutations, gene targeting technology, and several mutagenesis

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4 The abbreviations used are: DSB, DNA double-strand break; BAC, bacterial artificial chromosome; En, embryonic day; ESE, splicing enhancer motif; NE, nuclear envelope; NPC, nuclear pore complex; Pn, postnatal day; SC, synaptonemal complex; SNP, single-nucleotide polymorphism; SPB, spindle pole body; sqRT-PCR, semiquantitative RT-PCR; Tg, transgene.
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TABLE 1
Nucleotide sequences of PCR primers

| Primer Forward | Reverse |
|----------------|---------|
| H11 CCGCCCTCAAGGATCCCTTGTCC | TCTCTTGCCCCCTCCCCCTGT |
| Hspa2 CAGACGAGCGAAGTGTGAC | TTCCCTGGTCGTGTAATT |
| CCA1 AGTGGATGATGCTGTGAG | TTGGCGGGCGGTTAGAAAG |
| Sprm-1 GCCCTATTGTGTTTCCCCACTA | CCTCTCTGTGCTGCAAG |
| Hsp1l GTGAGTGGGCGCTGTAAG | TTCCGGCCTGACAGGCTGT |
| Gapdh AACCATTCCCCACCTTCCCT | CCTCTCTGTGCTGCAAG |
| Gapdh ATGGGATGATGCTGTGAG | TTGGCGGGCGGTTAGAAAG |
| D4Mit331 CTTAACCCCTCCCCACAC | AAAAGTCTGAGTACCCCTTCC |
| D4Mit168 GTCTTGGAAACCCACCCC | AGAAAGAAAGAAAAGACAGT |
| D4Mit20 CCAAAATAGCGGACTGACCC | GACAGCGAACACAGCACCAC |
| D4Mit31 AGCGTGGCTCCTGAGTCAACA | CTTCCGAGCCTCCCCCTGAA |
| D4Mit21 AGAGGAAAGCCTTTGAG | CGTTGGGCGGCTGGACAGT |
| D4Mit43 TCTTTCGAATGCTCCTGCTG | AGGAGAAGAGGCTTTGAG |
| D4Mit146 AAAATGGACAGGTTTTGGA | CAAAGAATCCGCTGGGCTG |
| D4Mit199 CTCAAGGCTGTCCTTAATACTG | TTAGCTGCAAGAAGTGACACAA |
| Tmem48-exon6_rTPCR CCCCTGCTGATACGCTGT | CTTTTGAGAGAGAGAGGCTG |
| Tmem48-exon6_genome GATCCGAGCTGTCCTT | CAAAGAATCCGCTGGGCTG |
| pET01_cDNAAlowen primer GATCCTGAGATG | AAGTCAAGTCTGAGAGG|
| pET01-exon A CCGAGGTTGATCCTGCTCT | GACAGGTTGATCCTGCTCT |
| pET01-exon B CCACATCGTCGTCATGCTGAA | GACAGGTTGATCCTGCTCT |
| Tmem48-exon6 CTCGACGCTGTCCTGCTGCTG | GACAGGTTGATCCTGCTCT |
| RP23-259013-SP6 CTGCCGAGTATGGTGAACAT | CCCAGGATCTGAGTGGATCTAA |
| RP23-259013-T7 CAACAACCGGTTCTCTGCTGAC | GAGTCCTCCCTCTAAGGAGT |
| Gli1 rs28147349 CCCAGGTTGATCCTGCTCT | GAGTCCTCCCTCTAAGGAGT |
| Yipf1 rs28147349 GCCATTGTTGATCCTGCTCT | GAGTCCTCCCTCTAAGGAGT |

EXPERIMENTAL PROCEDURES

Mice and Histological Preparations—The sks mice that were used in this study were obtained from the Mouse Mutant Resource at The Jackson Laboratory. The JF1/Ms strain was obtained from the National Institute of Genetics (Mishima, Japan). The sks mutant and normal mice were euthanized, and tissues were excised and fixed by immersion in Bouin’s fluid. After dehydration, tissues were embedded in paraffin wax and sectioned. After deparaffinization in xylene, sections were rehydrated and stained with Mayer’s hematoxylin and eosin. The protocols for the use of animals in this study were approved by the Animal Care and Use Committee of Okayama University.

Immunofluorescence Staining of Surface Spread Preparations of Testicular Cells—The surface spread preparations of testicular cells were prepared as described previously (7) with some modifications. Seminiferous tubules were dissociated in DMEM and centrifuged at 1800 rpm, and the collected cells were resuspended in DMEM. The cell suspension was spread onto the surface of the 0.5% NaCl solution, transferred to a silanized glass slide, and fixed with 2% paraformaldehyde containing 0.02% SDS. The surface-spread preparations were incubated with rabbit polyclonal anti-SCP3 antibody (8) that was diluted 1:500 and mouse monoclonal anti-bromodeoxyuridine antibody (9) that was diluted 1:200 and goat anti-mouse IgG-Alexa Fluor 488 (Upstate, Lake Placid, NY) that was diluted 1:300, and the preparations were incubated with rhodamine-conjugated donkey anti-rabbit IgG (AP182R, Chemicon International) that was diluted 1:200 and goat anti-mouse IgG-Alexa Fluor 488 (A-21211; Invitrogen) that was diluted 1:400 as secondary antibodies.

Semiquantitative (sq) RT-PCR Analysis—Total RNA samples of adult tissues, whole embryos of E10.5, testes of juvenile mice at various ages, ovaries of pubertal mice (21 days old), and each stage of the estrous cycle of adult mice were subjected to sqRT-PCR analyses. Total RNA from mouse tissues was extracted using TRIzol (Invitrogen) according to the manufacturer’s instructions. RNA preparations were treated with 10 units of DNase I (TaKaRa Bio, Shiga, Japan) and then purified. cDNA synthesis was performed by a reverse transcription reaction using Superscript III reverse transcriptase (Invitrogen) and oli-go(dT) primer (Invitrogen) according to the manufacturer’s instructions. The amounts of RNA were standardized according to the amplification of an internal control using the murine GAPDH primers (Table 1). The expression of stage-specific marker genes was examined by RT-PCR analysis by using primers that are listed in Table 1 as described in Dix et al. (9). sqRT-PCR was also performed to evaluate the expression pattern of the Tmem48 gene using primer pairs that are listed in Table 1. RT-PCR analysis was carried out in a reaction mixture containing a 100 nM concentration of each primer, a 100 μM concentration of each dNTP, and 0.25 unit of Taq polymerase (TaKaRa Neverland).
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Bio) with 25–30 cycles of amplification that consisted of 94 °C for denaturation, 53 °C-60 °C for annealing, and 72 °C for extension. The PCR products were electrophoresed on agarose gels.

**Linkage Mapping and Positional Cloning of the sks Gene**—To determine the chromosomal location of the sks locus, we performed linkage analysis using 532 F2 mice obtained by intercross of F1 mice derived from a cross between the sks and JF1/Ms mouse strains. To map the sks locus, genotypes of microsatellite makers of the F2 mice were determined by PCR in a reaction mixture containing 20 ng of genomic DNA, a 100 μM concentration of each primer for the microsatellite markers (Table 1), a 100 μM concentration of each dNTP, and 0.25 unit of Taq polymerase (TaKaRa Bio) with 35 cycles of amplification that consisted of 94 °C for denaturation, 53 °C-60 °C for annealing, and 72 °C for extension.

For sequence analyses of the positional candidate genes, entire coding regions of the candidate genes were amplified by sets of primers (Table 1), cloned in the pGEM T-easy vector (Promega), and sequenced by the dyeoxy chain termination method with a DNA sequencer HITACHI SQ5500.

For whole exome sequencing, size selected DNA (Pippin Prep; Sage Science) and Illumina Paired End libraries were constructed from genomic DNA using the Illumina-supplied protocol. Hybridization-based sequence capture was used to enrich the Illumina library for all mouse coding sequences (exome), Roche NimbleGen SeqCap EZ Mouse Exome Design (110624_MM9_Exome L2R_D02_EZ_HX1) was utilized according to the manufacturer’s protocols and as described by Fairfield et al. (10). After capture, enriched sequencing libraries used for cluster formation and paired end sequencing were done using Illumina HiSeq. Sequencing data were processed and aligned to the mouse genome (MGSC37, mm9) using a Burrows-Wheeler Alignmer, and SNPs/INDELs were called using SAMTools.

**In Vitro Splicing Assay**—The exon-trapping vector (Exontrap, pET01; MoBiTec, Göttingen, Germany) was used to investigate the splicing activity of exon 6 of the Tmem48 gene with the sks mutation. A 286-bp DNA fragment containing exon 6 and its flanking regions of the Tmem48 gene of the sks or wild-type allele was cloned into the pET01 vector. The clone carrying DNA from either wild-type (pET01-+) or sks alleles (pET01-sks) was transiently expressed in COS7 cells. COS7 cells were cultured in DMEM with 10% FCS and were seeded 24 h before transfection. COS7 cells were transfected with 1 μg of pET01-+ or pET01-sks using FuGENE 6 (Roche Diagnostics). Total RNA was extracted from COS7 cells 48 h after transfection, cDNA was synthesized with a primer for pET01-3′ transcriptional region, and RT–PCR was carried out using 2 sets of primers, pET01-exonA and pET01-exonB, or Tmem48-exon 6-specific primer and pET01-exonB (Table 1).

**Transgenesis Rescue of the sks Phenotypes**—A BAC clone, RP23-259013 (Invitrogen), containing an ~181-kb genomic fragment covering the Tmem48 gene that was cloned into the pBACE3.6 vector, was used for BAC transgenesis rescue experiments (see Fig. 8A). A DNA fragment containing the 181-kb BAC insert and flanking vector sequences was used for the pronuclear injection to generate the BAC transgenic mice containing the wild-type Tmem48 gene (see Fig. 8A). To genotype the transgenic animals, two sets of primers were used that amplified a 251-bp fragment of the T7 side of the pBACE3.6 vector-insert junction and a 289-bp fragment of the SP6 side of the pBACE3.6 vector-insert junction (see Fig. 8B and Table 1).

**Immunohistochemical Analysis Using Anti-TMEM48 Antibody**—A synthetic oligopeptide (HQKRLQQFLEFKE) that was derived from the C-terminal region of the mouse TMEM48 protein was used to immunize rabbits, and antibodies were purified from the serum of these rabbits. The specificity of the antibody was evaluated as follow. The Tmem48 protein fused with an N-terminal FLAG tag was expressed in COS7 cells by transfecting Tmem48/pCMV-Tg2. Lysates of transfected COS7 cells were electrophoresed through SDS-PAGE and transferred to a membrane, and the membrane was incubated with anti-TMEM48 or anti-FLAG M2 monoclonal antibody and goat anti-rabbit IgG HRP or goat anti-mouse IgG HRP as a secondary antibody. A neutralization test was also performed to test the antibody specificity. The antibody was preincubated with 0.5 mg/ml TMEM48 antigen before immunohistochemical staining. For immunofluorescence analysis of the transfected COS7 cells with anti-TMEM48 or anti-FLAG antibody, donkey anti-rabbit IgG rhodamine-conjugated (AP182R; Chemicon) and goat anti-mouse IgG-Alexa Fluor 488 (A-21211; Invitrogen) were used as secondary antibodies.

For the immunohistochemical analysis, tissue sections were incubated with PBS containing 0.3% H2O2 for 30 min and TNB buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% tyramide signal amplification blocking reagent) for 30 min at room temperature. The sections were then incubated with the rabbit anti-TMEM48 antibody (1:500) with 3% BSA in PBS overnight at 4 °C. The sections were incubated in series with HRP-conjugated anti-rabbit IgG (sc-2004, B0507; Santa Cruz Biotechnology) diluted with 3% BSA in PBS (1:1000), 2,4-dinitrophenylhydrazine (DNP) amplification reagents, anti-DNP-HRP antibody (PerkinElmer Life Sciences), and 3,3’-diaminobenzidinetetrahydrochloride chromogen (Dako, Kyoto, Japan). The sections were counterstained with Mayer’s hematoxylin and were examined by light microscopy.

**Western Blot Analysis**—Mouse testes were lysed in the radioimmunoprecipitation assay buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, and 1 mM protease/phosphatase inhibitors). Equal amounts of the extracted proteins of the sks/sks and +/-/+ mice were separated by 7.5% SDS-PAGE and transferred to an Immobilon-P membrane (Millipore). The filters were incubated with anti-TMEM48 antibody that was diluted in 2% blocking buffer. The filters were washed with PBS-Tween 20 and then were incubated with HRP-conjugated goat anti-rabbit IgG. The HRP signal was detected using the Amersham Biosciences ECL Advance Western blotting Detection Kit (GE Healthcare) according to the manufacturer’s instructions.

**RESULTS**

**Spermatogenesis in sks Mice Is Interrupted at the Pachytene Stage of Meiotic Prophase**—The testes of adult sks/sks mice were smaller than those of normal littermate animals, but the
sizes of other male reproductive organs, including the epididymis and seminal vesicle, showed no clear difference (Fig. 1A). The testis weights of adult sks/sks mice (0.045 ± 0.004 g) were less than one-fourth of those of normal mice (0.206 ± 0.046 g). Histological examination of the testes showed that most seminiferous tubules of the sks/sks mice contained germ cells at all stages of development up to pachytene stage, but neither spermatids nor spermatozoa were observed, and significant portions of seminiferous tubules were vacuolized (Fig. 1B) as reported previously (6). To further investigate the underlying spermatogenesis defect in the mutant mouse, we compared testis sections of sks/sks mice and normal littermates during the first wave of spermatogenesis at postnatal day (P) 14, 16, 18, 22, and 35 (Fig. 2) when spermatogenesis is synchronized (11). The seminiferous tubules of the mutant and normal mice showed an organized progression of cell types from spermatogonia at the periphery to leptotene, zygotene, or early-mid pachytene spermatocytes at the innermost layers at P14, and no significant difference was observed in the two genotypes (Fig. 2, A and B). At P16, mid-late pachytene spermatocytes and a few meiotic metaphase cells were apparent in the seminiferous tubules of the normal mice (Fig. 2C), and spermatocytes completed meiosis at P18 with the appearance of the round spermatids (Fig. 2E). However, although a few mid-late pachytene spermatocytes were observed, neither metaphase cells nor round spermatids were observed in the testes of the mutant mice at these stages (Fig. 2, D and F). More substantial differences appeared at later stages. The seminiferous tubules of the normal mice were filled with round spermatids at P22 (Fig. 2G) and showed the release of mature spermatozoa into the lumen at P35 (Fig. 2I), but the seminiferous tubules of the mutant mice contained no postmeiotic germ cells, and cells with condensed nuclei and multinucleated cells were frequently observed (Fig. 2, H and J). These findings indicate that the differentiation of most mutant germ cells is interrupted at mid to late pachytene stage of meiosis, although a few meiotic metaphase cells were observed in the seminiferous tubules of the adult mutant mice (Fig. 1B).

Expression of Stage-specific Marker Genes of Spermatogenesis—We examined the expression of the genes that have a stage-specific expression pattern during spermatogenesis by sqRT-PCR analysis to determine when the germ cell development was interrupted in the sks/sks mice. RT-PCR analysis of adult testis of the mutant mice revealed normal expression of the Hist1H gene, significantly reduced Hspa2 and Ccna1 gene expression, and no or trace levels Pou5f2, Hsp11, and Tnp1 gene expression.
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expression of the genes was examined at stages later than late pachytene stage in normal mice was not detected in the sks/sks mice. These results confirmed that spermatogenesis of the sks/sks mice is arrested at the late pachytene stage.

Surface Spread Preparation of Spermatocytes—During prophase of meiosis, a homologous chromosomes pair and a SC is formed between the homologous chromosomes. We examined whether pairing of homologous chromosomes and the formation of SC proceeded normally in the sks/sks spermatocytes by immunofluorescence staining using antibodies against SCP3 and γH2AX. SCP3 is a component of the lateral element of SC, and γH2AX is a phosphorylated histone variant that accumulates on the DSB sites and can be used as a maker for asynapsis. The patterns of γH2AX staining in the sks/sks spermatocytes at the leptotene to zygotene stage were similar to those of the normal mice. However, ~40% of the mutant spermatocytes at the pachytene stage showed apparently different γH2AX staining patterns. As shown in Fig. 4, a γH2AX foci was restricted to the X-Y body in the normal spermatocytes, which represented an unpaired region of the X and Y chromosomes. However, defused γH2AX foci other than the X-Y body were frequently observed in the sks/sks spermatocytes which indicates unpaired DSBs in autosomes. These spermatocytes with the defused γH2AX foci also showed partial asynapsis of homologous chromosomes that was indicated by SCP3 signals (Fig. 4, A and B). These findings confirmed defects of homologous chromosome panning and synapsis in the spermatocytes of the sks/sks mice as reported previously (6).

Linkage Mapping of the sks Locus—We performed fine mapping of the sks locus by linkage analysis to identify the gene responsible for the sks mutation. A total of 532 F2 progeny were obtained from the cross between sks and JF1/Ms mice. As the sks locus has been mapped to a 8-Mb interval of the distal region of mouse chromosome 4, we genotyped 9 microsatellite markers in this region in the 63 sks/sks and 469 unaffected mice. The segregation data of these markers indicated the localization of the sks gene to an ~0.3-Mb interval between D4Mit31 and D4Mok22 in which nine genes were localized (Fig. 5).

Characterization of the Candidate Genes in the sks Locus—We sequenced the entire coding regions of these nine genes: Tceanc2, Tmem59, Ldbrad1, Lrrc42, Hspb11, Dio1, Yipf1, Tmem48, and Gils1. Comparing the nucleotide sequences of these candidate genes in the sks mutant and normal mice revealed no nucleotide sequence differences in these genes except for the Tmem48 gene. However, a remarkable difference was observed in the transcripts of the Tmem48 gene. As shown in Fig. 6A, the amplification of cDNA spanning exon 6 of the gene by RT-PCR yielded a fragment with the expected size (287 bp) in the normal mouse, but the fragment from the mutant mouse was ~100 bp shorter than the expected size. Comparing the nucleotide sequence of these two transcripts revealed that a 109-bp sequence corresponding to exon 6 of the Tmem48 gene was deleted in the transcripts of the mutant mice. RT-PCR analysis of Tmem48 in several tissues showed that the deletion of exon 6 of Tmem48 transcripts was observed in all of the sks/sks tissues but not the normal tissues, indicating that the deletion of exon 6 is not caused by tissue-specific alternative splicing. The deletion of exon 6 creates a frameshift mutation resulting in premature termination at codon 225. By Western blot analysis using anti-TMEM48 antibody, we confirmed that...
no mature TMEM48 protein was present in the tissues of sks/sks mice, whereas a protein of 65 kDa was observed in the normal mice (Fig. 6D). To determine the cause of the deletion, we compared the genomic sequence of Tmem48 in the sks mutant and normal mice. Neither deletion of the region containing exon 6 nor mutation at the splicing donor or acceptor sites of the exon was observed, but a nucleotide substitution of GG to TT was found in exon 6 (Fig. 6B). Although the GG to TT substitution is predicted to result in a missense mutation of arginine (R) to leucine (L), splicing pattern of the Tmem48 gene in the sks mutant and wild-type alleles. D, Western blot analysis of testis extracts using anti-TMEM48 antibody indicating the absence of TMEM48 protein in the sks mutant. The arrowhead indicates a specific band of TMEM48 with a molecular mass of 65 kDa. E, Western blotting of FLAG-tagged Tmem48 showing specificity of the antibody. Anti-TMEM48 antibody showed a specific band with a molecular mass of 65 kDa in the lysates of COS7 cells transfected with Tmem48/pCMV-Tg2. The same band was also detected by the anti-FLAG antibody. Lanes 1, COS7 with Tmem48/pCMV-Tg2. Lanes 2, intact COS7 without vector.

To confirm that the GG to TT substitution of Tmem48 gene is unique to sks mice and no other gene of the candidate region has nucleotide lesion in the sks mice, we performed whole exome sequencing of sks mouse. Using the data generated from high throughput sequencing of whole exomes, we performed a genome-wide comparison of coding sequence from sks and a strain background matched control (A/J). This comparison allowed us to eliminate from consideration all strain background-specific SNPs. We also compared these data with a large set (250) of whole exome datasets from unrelated spontaneous mutants and control inbred strains generated by the Jackson Laboratory Mutant Mouse Resource. As result, we confirmed that the GG to TT nucleotide substitution at 4:107053225–107053226 (MGSC37, mm9) in exon 6 of Tmem48 was unique to sks and not found in any other strains of mice. We also confirmed the absence of any other lesion in the remaining genes in the candidate region including Tceanc2, Tmem59, Ldrlad1, Lrcc42, Hspb11, Dio1, Yipf1, and Gils1. These findings strongly suggested that the GG to TT substitution is the cause of the deletion of exon 6 in Tmem48 gene of sks mice.

In Vitro Assay for the Splicing of the Mutant sks Gene—To confirm the relation between the nucleotide substitution in exon 6 and the deletion of exon 6 in the sks mouse, we examined the effect of the nucleotide substitution on the splicing of the gene by an in vitro splicing assay using the pET01-sks and pET01+ vector containing the mutant and wild-type exon 6 and a part of introns 5 and 6 into pET01, respectively (Fig. 7A). COS7 cells were transfected by these vectors, and the splicing pattern of transcripts of the transfected COS7 cells was analyzed by RT-PCR. As shown in Fig. 7B, whereas the pET01+ produced a significant amount of transcripts containing exon 6,
no transcripts containing exon 6 were produced from pET01-sks. These findings demonstrated that the GG to TT nucleotide substitution of the Tmem48 gene is responsible for the deletion of exon 6 that was caused by the aberrant splicing.

Transgenesis Rescued the sks Phenotypes—To confirm that the nucleotide substitution of the Tmem48 gene is responsible for the phenotypes of the sks/sks mice, we generated transgenic mice with a wild-type Tmem48 transgene. The transgenic mice were generated by pronuclear injection of the genomic fragment obtained from a BAC clone containing the wild-type Tmem48 gene. As shown in Fig. 8A, the 181-kb BAC RP23 insert indicated as the gray bar contains entire exons, introns, and the promoter region of the Tmem48 gene, but it contains only a 3′ and 5′ part of the flanking genes, Yipf1 and Glis1, respectively. Therefore, the functional gene in this BAC fragment is only the Tmem48 gene. The resultant Tmeme48 transgene-positive (Tg+) transgenic mice were crossed with sks/+ mice, and the sks/sks;Tg+ mice were obtained by a cross between the sks/+;Tg+ and sks/+ mice. All resultant sks/sks; Tg+ mice are viable and morphologically indistinguishable from their normal littermates, and the no skeletal abnormality was observed (Fig. 8, C and E). Both males and females showed normal fertility, and no abnormality in the spermatogenesis was observed by histological examination (Fig. 8D). Therefore, the wild-type Tmem48 gene completely rescued the phenotypes of the sks/sks mice, demonstrating that the nucleotide substitution of Tmem48 is responsible for the phenotypes of the sks mice.

Expression Analysis of the Tmem48 Gene—Expression analysis by sqRT-PCR of various tissues of wild-type mice revealed that the Tmem48 gene was expressed in several mouse tissues, including testis, ovary, and E10.5 embryos (Fig. 9A). Subsequently, Tmem48 expression in testes of P2 to P32 juvenile mice during the first wave of spermatogenesis was examined to determine whether the Tmem48 gene is expressed at the specific stages of spermatogenesis. As a result, Tmem48 expression was detected in all developmental stages of testes (Fig. 9B), indicating no stage-specific expression of Tmem48 in differentiating germ cells. We also examined Tmem48 expression in ovaries of juvenile mice (3 weeks old) and different stages of the estrous cycle of adult mice; we found expression in the ovaries of juvenile and adult mice, and the expression levels changed depending on the stages: the highest expression level was detected in the diestrus stage, and the lowest level was detected in the metestrus stage (Fig. 9C).

Immunohistochemical Analysis—The localization of the TMEM48 protein in the testes of the wild-type mice was examined by immunohistochemistry using anti-TMEM48 antibody. The results revealed the stage-specific localization of TMEM48 in testicular germ cells. As shown in Fig. 10, we classified the stages of the cycle of the seminiferous epithelium according to Russell et al. (12). Positive immunological reactivity for
TMEM48 was observed in the cytoplasm of round and elongating spermatids in the stage I seminiferous epithelium, round spermatids in stages II–VI, and round or elongating spermatids and pachytene or diplotene spermatocytes in stages VII–XI (Fig. 10A). In stage XII, immunological reactivity was observed in elongating spermatids but not in the spermatocytes in metaphase (Fig. 10A). In contrast, no immunological reactivity was observed in other types of testicular germ cells, including spermatogonia, leptotene, and zygotene spermatocytes, elongated spermatids, and somatic cells like Leydig and Sertoli cells (Fig. 10A). Although the localization of TMEM48 on the nuclear envelope is not clear in the immunohistochemical staining of the testis sections, clear localization of TMEM48 on the nuclear envelope was observed in cultured COS7 cells that were transfected with the Tmem48 gene (Fig. 10D). These results indicate that TMEM48 was specifically expressed in the cytoplasm and

FIGURE 8. Rescue of sks phenotypes by transgenic mice with wild-type Tmem48 gene. A, physical map of the genomic region including the Tmem48 gene. The positions of the Tmem48 gene and the flanking Yipf1 and Glis1 genes are indicated. SNPs Yipf1rs1 and Glis1rs1 were used to distinguish the endogenous and transgenic Tmem48 genes. B, genotyping of the endogenous and transgenic Tmem48 genes of transgenic mice that were crossed with sks mice. The upper and lower bands of Tmem48-exon 6 represent the sks and wild-type Tmem48 allele, respectively. The upper and lower bands of Yipf1rs1 and Glis1rs1 SNPs are associated with the sks and wild-type allele, respectively. RP23-SP6 and RP23T7 are PCR fragments represent SP6 and T7 sides of the pBACe3.6-insert junctions, respectively. C, gross appearance of the transgenic sks mice, showing rescue of sks phenotypes including small body size, kinky tail, and small testis in sks/sks; Tg+/H11001. D, histology of testes of the transgenic sks mice showing normal spermatogenesis in sks/sks; Tmem48TG+/H11001. Scale bars, 100 μm.
A Mutation of Tmem48 Causes Gametogenesis Defects in Mice

In this study, we identified a nucleotide substitution of GG to TT in exon 6 of Tmem48 that is accompanied by defective splicing that excludes exon 6 in the Tmem48 transcripts of sks mutant mice. We also demonstrated that the GG to TT substitution is sufficient to cause skipping of exon 6 in an in vitro splicing assay. These findings indicate that the nucleotide substitution in exon 6 is responsible for the deletion of exon 6 in the transcripts. Various nucleotide substitutions have been reported to cause human genetic defects with splicing abnormalities (13). Most of these nucleotide substitutions directly affect canonical splicing signals, including the 5’ splicing donor, branching, and/or 3’ splicing acceptor sites. Nucleotide substitutions that create an ectopic splice site have also been reported (13, 14). For example, a G to A missense mutation in exon 5 of the PMM2 gene, which is associated with carbohydrate-deficient glycoprotein syndrome type Ia, causes a deletion of exon 5 in the transcripts of the gene (15). In these exon-skipping mutations, the nucleotide substitutions disrupt putative ESEs. ESEs are cis-acting elements that stimulate splicing (13) and are thought to serve as binding sites for a family of splicing factors such as SF2/ASF, SC35, SRp40, and SRp50 (13). Therefore, the nucleotide substitution of the sks mouse was suggested to disrupt ESEs in exon 6 of the Tmem48 gene.

ESEs have consensus motifs, and software that identifies putative ESEs and evaluates whether exonic mutations disrupt the ESEs has been developed. We examined whether the nucleotide substitution of the Tmem48 gene disrupts ESEs in exon 6 using these programs. ESEfinder predicted that the nucleotide substitution of GG to TT disrupted SF2/ASF and SRp40 binding motifs of exon 6. We also evaluated the effect of the nucleotide substitution using Rescue-ESE v1.0, which predicts the ESEs based on a list of hexamer motifs that are enriched in exons (17). Rescue-ESE predicted that two ESEs that are found in exon 6 of wild-type Tmem48 are missing in the sks mutant. These results strongly suggested that the exon skipping of the Tmem48 gene is caused by disrupted ESEs in exon 6 of the Tmem48 gene by the GG to TT nucleotide substitution.

The Tmem48 gene comprises 18 exons and encodes a transmembrane protein of 673 amino acids with six transmembrane domains. Tmem48 is homolog of Ndc1, a nuclear pore complex (NPC) protein of Saccharomyces cerevisiae (18, 19). The Ndc1 protein serves as a membrane-anchored protein to connect the NPC to the nuclear envelope (NE) (20, 21). Recently, the human NDC1 protein was also reported to be essential for NPC assembly and NE formation (20, 21). The NPC is a large protein complex that crosses the NE and is composed of approximately 30 nucleoporin proteins (22). NDC1 is known to interact with the nucleoporin NUP53, which is tightly associated with the nuclear membrane and physically interacts with other NPC proteins, including NUP155 (20, 23). In the Drosofila mutant of the Nup155 homologous gene, spermatogenesis arrested at prophase of meiosis I, the transition to metaphase did not occur, and oogenesis failed to progress into the vitellogenic stage; this resulted in sterility in both sexes (24). Furthermore, Thomas and Botstein have reported that the budding yeast NDC1 mutant could not produce normal asci, which was caused by the failure of chromosome separation at meiosis (18). In this study, we found that the spermatogenesis of sks mice arrested at prophase of meiosis I, and TMEM48 protein expression was observed in spermatocytes at pachytene and diplotene stages of meiotic prophase. Furthermore, the sks mice showed defects in the synopsis of homologous chromosomes during meiotic prophase. These phenotypic similarities between of the sks mouse and the fruit fly and yeast with mutations in NPC genes suggested that the NPC has specific functions for meiosis, particularly in chromosome synopsis and separation. The major function of the NPC is thought to be the transport of water-soluble molecules across the NE, which

FIGURE 9. Expression of the Tmem48 gene in mouse tissues and developmental stages. A, expression of the Tmem48 gene in mouse brain (Br), heart (He), lung (Lu), liver (Li), spleen (Sp), stomach (St), intestine (In), kidney (Ki), skeletal muscle (Mu), bone marrow (BM), skin (Sk), ovary (Ov), and testis (Te) of adult mouse, and whole embryos (Em) at E10.5. B, expression of the Tmem48 gene in mouse testis during the first wave of spermatogenesis. The expression patterns of Hspa2, which is expressed after spermatocytes reach the zygotene stage, and Hsp41, which is expressed after the round spermatid stage, are indicated for comparison. C, expression of the Tmem48 transcripts in ovary at different stages of the estrous cycle. Ju, juvenile (3 weeks after birth); Pro, proestrus; Es, estrus; Me, metestrus; and Di, diestrus. The amounts of RNA were normalized according to the expression level of the Gapdh gene.

A

B

C
includes the export of RNA molecules from the nucleus to the cytoplasm and the import of particular nuclear proteins into the nucleus (25). The function of NPC in meiosis and gametogenesis is unclear, but the nuclear import of certain proteins, including those comprising chromatin and the synaptonemal complex, is required for the progression of meiosis. Therefore, the mutation of Tmem48 in sks mice might affect the transport of particular molecules that are essential...
for the synopsis of homologous chromosomes during meiosis through the NPC.

The *S. cerevisiae* Ndc1 and the *Schizosaccharomyces pombe* homolog of the NDC1 protein localize not only to the nuclear pore but also the spindle pole body (SPB) and is known to be essential for the formation of SPBs (18, 26). The SPB is the microtubule-organizing center in yeast cells and is functionally equivalent to the centrosome in higher eukaryotes (27, 28). Several lines of genetic and cytological evidence suggest that SPBs or microtubules interact with the telomeres of chromosomes during prophase of meiosis I because all telomeres transiently gather at the NE and cluster around the SPB in fission yeast or the centrosome in mammals (29). This polarized chromosome arrangement is called the meiotic telomere clustering or the bouquet formation (29, 30). The bouquet formation coincides with defective synapsis that was observed in the sks mice could be caused by defective bouquet formation during meiosis.

Although the molecular mechanisms controlling mammalian gametogenesis are not fully understood because of its complexity, this is the first report to demonstrate that an NPC protein is essential for the proper progression of meiosis during mammalian gametogenesis. Additionally, sks mice, which show defects in gametogenesis that are caused by a mutation in the Tmem48 gene, will be a useful model for studying the function of the NPC in meiosis and gametogenesis.

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REFERENCES

1. Handel, M. A., and Schimenti, J. C. (2010) Genetics of mammalian meiosis: regulation, dynamics and impact on fertility. *Nat. Rev. Genet.* 11, 124–136
2. Matzuk, M. M., and Lamb, D. J. (2002) Genetic dissection of mammalian fertility pathways. *Nat. Cell Biol.* 4, 541–49
3. Akiyama, K., Akimaru, S., Asano, Y., Khalaj, M., Kiyosu, C., Masoudi, A. A., Takahashi, S., Katayama, K., Tsuji, T., Noguchi, J., and Kunieda, T. (2008) A newENU-induced mutant mouse with defective spermatogenesis caused by a nonsense mutation of the syntaxin 2/epimorphin (Sct2/Epim) gene. *J. Reprod. Dev.* 54, 122–128
4. La Salle, S., Palmer, K., O’Brien, M., Schimenti, J. C., Eppig, J., and Handel, M. A. (2012) Spats2a2, a novel vertebrate-specific gene, is required for meiotic progress in mouse germ cells. * Biol. Reprod.* 86, 45
5. Sun, F., Palmer, K., and Handel, M. A. (2010) Mutation of Epig3, encoding a eukaryotic translation initiation factor, causes male infertility and meiotic arrest of mouse spermatocytes. *Development* 137, 1699–1707
6. Handel, M. A., Lane, P. W., Schroeder, A. C., and Davison, M. T. (1988) New mutation causing sterility in the mouse. *Gamete Res.* 21, 409–423
7. Noguchi, J., Ozawa, M., Nakai, M., Somfai, T., Ikukchi, K., Kaneho, K., and Kunieda, T. (2008) Affected homologous chromosome pairing and phosphorylation of tests specific histone, H2AX, in male meiosis under FKBP6 deficiency. *J. Reprod. Dev.* 54, 203–207
8. Chuma, S., and Nakatsuji, N. (2001) Autonomous transition into meiosis of mouse fetal germ cells in vitro and its inhibition by gp130-mediated signaling. *Dev. Biol.* 229, 468–479
9. Dix, D. J., Allen, I. W., Collins, B. W., Poorman-Allen, P., Mori, C., Blizard, D. R., Brown, P. R., Goulding, E. H., Strong, B. D., and Eddy, E. M. (1997) HSP70–2 is required for desynapsis of synaptonemal complexes during meiotic prophase in juvenile and adult mouse spermatocytes. *Development* 124, 4595–4603
10. Fairfield, H., Gilbert, G. J., Barter, M., Corrigan, R. R., Curtian, M., Ding, Y., D’Ascanzo, M., Gerhardt, D. J., He, C., Huang, W., Richmond, T., Rowe, L., Probst, F. J., Bergstrom, D. E., Murray, S. A., Bult, C., Richardson, J., Kile, B. T., Gut, I., Hager, J., Sigurdsson, S., Mauceli, E., Di Palma, F., Lindblad-Toh, K., Cunningham, M. L., Cox, T. C., Justice, M. J., Specter, M. S., Lowe, S. W., Albert, T., Donahue, L. R., Jeddeloh, J., Shendure, J., and Reinholdt, L. G. (2011) Mutation discovery in mice by whole exome sequencing. *Genome Biol.* 12, R86
11. Bellvé, A. R., Cavicchia, J. C., Millette, C. F., O’Brien, D. A., Bhatnagar, Y. M., and Dym, M. (1977) Spermatogenic cells of the prepubertal mouse: isolation and morphological characterization. *J. Cell Biol.* 74, 68–85
12. Russell, L. D., Ettlin, R. A., Sinha Hihim, A. P., and Clegg, E. D. (1990) *Histological and Histopathological Evaluation of the Testis*, Cache River Press, Clearwater, FL
13. Cartegni, L., Chew, S. L., and Krainer, A. R. (2002) Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat. Rev. Genet.* 3, 285–298
14. Blencowe, B. J. (2000) Exonic splicing enhancers: mechanism of action, diversity and role in human genetic diseases. *Trends Biochem. Sci.* 25, 106–110
15. Vuillaume-Barrot, S., Barner, A., Cuer, M., Durand, G., Grandchamp, B., and Seta, N. (1999) Characterization of the 415G>A (E139K) PPM2 mutation in carbohydrate-deficient glycoprotein syndrome type Ia disrupting a splicing enhancer resulting in exon 5 skipping. *Hum. Mutat.* 14, 543–544
16. Cartegni, L., Wang, J., Zhu, Z., Zhang, M. Q., and Krainer, A. R. (2003) ESEfinder: a web resource to identify exonic splicing enhancers. *Nucleic Acids Res.* 31, 3568–3571
17. Fairbrother, W. G., Yeo, G. W., Yeh, R., Goldstein, P., Mawson, M., Sharp, P. A., and Burge, C. B. (2004) RESCUE-ESE identifies candidate exonic splicing enhancers in vertebrate exons. *Nucleic Acids Res.* 32, W187–190
18. Thomas, J. H., and Botstein, D. (1986) A gene required for the separation of chromosomes on the spindle apparatus in yeast. *Cell* 44, 65–76
19. Winey, M., Hoyt, M. A., Chan, C., Goetsch, L., Botstein, D., and Byers, B. (1993) NDC1: a nuclear periphery component required for yeast spindle pole body duplication. *J. Cell Biol.* 122, 743–751
20. Mansfeld, J., Güttiger, S., Hawrylik-Gara, L. A., Panté, N., Mall, M., Galy, V., Haselmann, U., Mühlhäuser, P., Wozniak, R. W., Matij, I. W., Kutay, U., and Antonin, W. (2006) The conserved transmembrane nucleoporin NDC1 is required for nuclear pore complex assembly in vertebrate cells. *Mol. Cell. Biol.* 22, 93–103
21. Stavr, F., Hülsmann, B. B., Spang, A., Hartmann, E., Cordes, V. C., and Görlich, D. (2006) NDC1: a crucial membrane-integral nucleoporin of metazoan nuclear pore complexes. *J. Cell Biol.* 173, 509–519
22. Chatel, G., and Fahrenkrog, B. (2011) Nucleoporins: leaving the nuclear pore complex for a successful mitosis. *Cell. Signal.* 23, 1555–1562
23. Hawrylik-Gara, L. A., Shibuya, E. K., and Wozniak, R. W. (2005) Vertebrate assembly of a Nup93-containing complex. *Mol. Biol. Cell* 16, 2382–2394
24. Gigliotti, S., Calliani, G., Andone, S., Riparbelli, M. G., Pennas-Alonso, H., Hoffmann, G., Grazianni, F., and Malva, C. (1998) Nup154: a new Drosophila gene essential for male and female gametogenesis, is related to the Nup155 vertebrate nucleoporin gene. *J. Cell Biol.* 142, 1195–1207
25. Strambio-De-Castillia, C., Niepel, M., and Rount, M. P. (2010) The nuclear pore complex: bridging nuclear transport and gene regulation. *Nat. Rev. Mol. Cell Biol.* 11, 490–501
26. West, R. R., Vaisberg, E. V., Ding, R., Nurse, P., and McIntosh, J. R. (1998) cut17 †: a gene required for cell cycle-dependent spindle pole body anchoring in the nuclear envelope and biopolar spindle formation in *Schizosaccharomyces pombe*. *Mol. Biol. Cell* 9, 2839–2855
27. Byers, B., Shriver, K., and Goetsch, L. (1978) The role of spindle pole bodies and modified microtubule ends in the initiation of microtubule assembly in *Saccharomyces cerevisiae*. *J. Cell Sci.* **30**, 331–352

28. Hyams, J. S., and Borisy, G. G. (1978) Nucleation of microtubules in vitro by isolated spindle pole bodies of the yeast *Saccharomyces cerevisiae*. *J. Cell Biol.* **78**, 401–414

29. Harper, L., Golubovskaya, I., and Cande, W. Z. (2004) A bouquet of chromosomes. *J. Cell Sci.* **117**, 4025–4032

30. Scherthan, H. (2001) A bouquet makes ends meet. *Nat. Rev. Mol. Cell Biol.* **2**, 621–627

31. Zickler, D., and Kleckner, N. (1998) The leptotene-zygotene transition of meiosis. *Annu. Rev. Genet.* **32**, 619–697