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Original Article

CMTM2 is essential for spermiogenesis in mice

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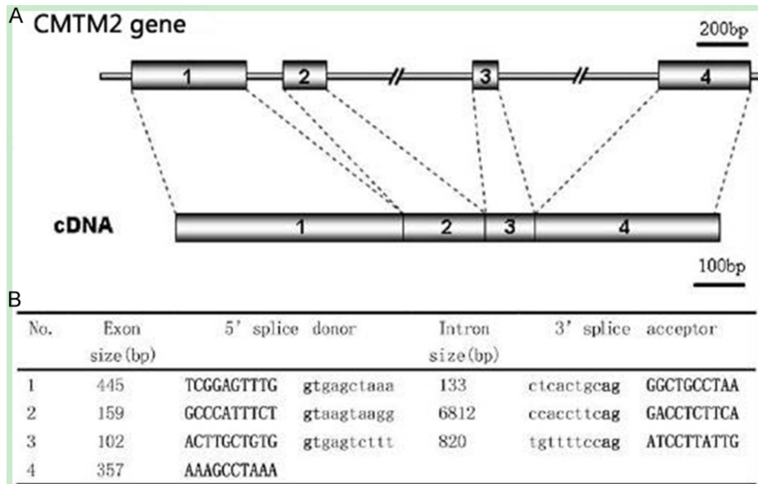
Abstract: Objective: This study is to investigate whether CKLF-like MARVEL transmembrane domain-containing protein 2 (CMTM2) is involved in spermatogenesis in mice. CMTM2 is highly expressed in testis, and could possibly a potential spermagogenesis specific gene. Methods: CMTM2-deficient mouse model was generated. Northern, RT-PCR and Western blotting analysis were performed on total RNA derived from wild-type (WT, *CMTM2*$^{+/+}$) and *CMTM2*$^{+/-}$ (heterozygote) and *CMTM2*$^{-/-}$ (homozygote) mice to examine the CMTM2 level. The number of litters and the number of pups were counted and pregnancy rates calculated. The motility and morphology of the sperm and the histology of testes were analyzed. Serum testosterone and FSH concentrations were also measured. Standard t-tests (Excel, Microsoft, Redmond, WA, USA) were used and standard error of means were calculated. Results: CMTM2 is highly expressed in a finely regulated pattern in the mouse testis during spermatogenesis. The body weight of adult mice with CMTM2 deficiency was not significantly different from that of wild type mice. No obvious anatomical or behavioral abnormalities were observed. The testes of *CMTM2*$^{-/-}$ were smaller than that of *CMTM2*$^{+/+}$ mice. Female CMTM2 null mice are fertile, indicating that CMTM2 is not required for female gametogenesis. The *CMTM2*$^{-/-}$ mice produced virtually no sperm, and *CMTM2*$^{+/-}$ mice sperm count showed a significant decline. The hormone levels are not significantly different. The *CMTM2*$^{-/-}$ male mice are sterile due to a late, complete arrest of spermiogenesis. The organized architecture of the seminiferous epithelium of the seminiferous tubules seen in *CMTM2*$^{+/+}$ mice was lost in *CMTM2*$^{-/-}$ mice. Conclusions: This study suggests CMTM2 is not required for embryonic development in the mouse but is essential for spermiogenesis.

Keywords: CMTM2, sperm, gene knockout mouse model, spermiogenesis

Introduction

Reproduction and development is one of the most active topics in the field of biomedical research in recent years. Despite its importance in human health, the pathogenesis of male infertility remains poorly understood [1]. A multitude of conditions can cause male infertility, such as congenital malformations, exposure to polluted environment, genetic and endocrine disorders, and infectious, inflammatory and immunologic conditions [2]. The post-meiotic development, also known as spermiogenesis, is dependent on the accurate expression of a large number of testis-specific genes, the disruption of which often results in spermatogenic defects and male infertility [3-5].

Previously, it has been reported that CMTM2 (CKLF-like MARVEL transmembrane domain-containing protein 2) is possibly associated with spermatogenesis [6]. CMTM2 is one of the members of CMTM family which is previously called chemokine-like factor superfamily (CKLFSF) (**Figure 1**). It is a novel protein family that provides a structural and functional link between chemokines and members of the transmembrane 4 super family (TM4SF) [7, 8]. Interestingly, most members of the CMTM family have higher expression levels in testis, indicating they might play systemic roles in spermatogenesis. Among members of the CMTM family, CMTM2 is highly expressed in testis (**Figure 2**) [9]. In addition, the importance of CMTM2 in spermatogenesis was suggested by a close correlation between CMTM2 abnormal expression and a spermatogenesis defect [6]. With the aggravation of the spermatogenesis defect, the CMTM2 positive cell numbers and mRNA level decreased significantly with no expression in the testes of patients with Sertoli Cell Only Syndrome (SCOS), which is character-



No.	Exon size (bp)	5' splice donor		Intron size (bp)	3' splice acceptor	
1	445	TCGGAGTTTG	gtgagctaaa	133	ctcactgcag	GGCTGCCTAA
2	159	GCCCATTTCT	gtaagtaagg	6812	ccaccttcag	GACCTCTTCA
3	102	ACTTGCTGTG	gtgagtcttt	820	tgttttccag	ATCCTTATTG
4	357	AAAGCCTAAA				

Figure 1. Genomic structure of human CMTM2. A. Schematic map of the human CMTM2 gene. B. Sizes of exons and introns, and splice junction sequences of the human CMTM2 gene.

ized histologically by a complete loss of the germinal epithelium in human testicular tubules [6, 10]. This correlation provided compelling evidence for a crucial role of CMTM2 in spermatogenesis.

In this study, we have established CMTM2 knockout mouse model and observed its effects on male fertility from the aspects of reproductive phenotype, sperm quality, fertile function and testis changes.

Materials and methods

Animals

The CMTM2 null mice were a generous gift from Dr. Kite Brandes at the Washington University in St. Louis. These mice were both on C57BL/6 backgrounds and thus age-matched but non-related controls from this strain were used. Mice were housed according to Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

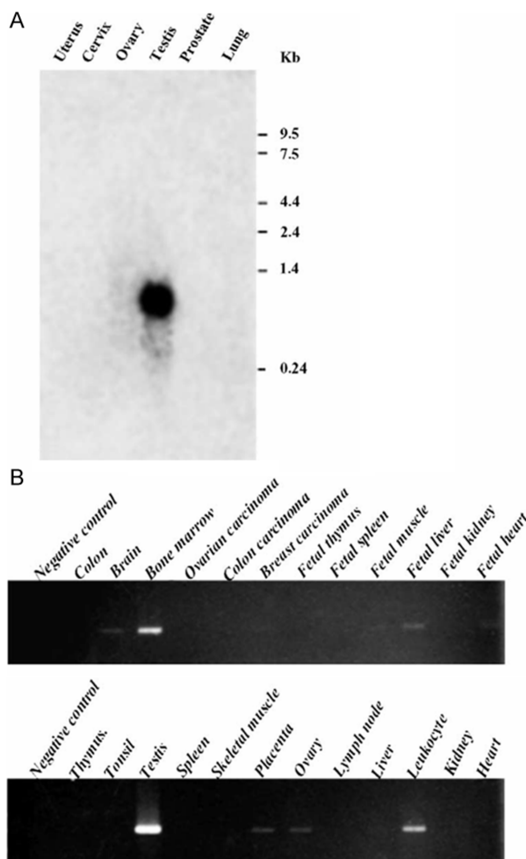


Figure 2. Tissue distribution of human CMTM2 mRNA. The expression of human CMTM2 was PCR-detected in a human multiple tissue cDNA panel, and analyzed by agarose gel electrophoresis. The cDNA samples present within each MTC panel were pre-normalized using several housekeeping genes.

Dissection techniques

Adult males 8-12 weeks of age were used for this study. Intraperitoneal ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively) mixture was used for anesthesia. Prior to euthanasia, a midline incision was made from the xiphoid to pubis, and the abdominal contents were carefully inspected with the assistance of a dissecting microscope or × 2.5 loupe dissecting lenses. Animals then were euthanized using intracardiac pentobarbital (100 mg/kg).

Analysis of gene expression

Northern and RT-PCR analysis was performed on total RNA derived from wild-type (WT, *CMTM2*$^{+/+}$) and *CMTM2*$^{+/-}$ (heterozygote) and *CMTM2*$^{-/-}$ (homozygote) mice (a list of RT-PCR primers is available on request) using standard protocols. Control PCR reactions were performed on *CMTM2*$^{-/-}$ RNA in the absence of reverse transcriptase. Immunoblots were performed on testis protein extracts using the monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Testes histology

Mice testes were fixed in Bouin's fixative immediately after euthanasia. Specimens were cut at 4 μm thickness and stained with hematoxylin and eosin for examination by light microscopy. Specific cell counts were performed in each testicle in the seminiferous epithelium of five round-shaped seminiferous tubules, i.e. cells were counted in 10 tubules per mouse. Two independent, blind observers performed the cell counts.

Western blotting analysis

Western blot analysis of endogenous proteins from organ tissues was performed as described previously [6]. The following antibodies were used: rabbit monoclonal CMTM2 antibody (Cell Signaling Technology, Inc., Beverly, MA, USA), rabbit monoclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Sigma-Aldrich) and goat anti-rabbit IgG-HRP antibody (Sigma-Aldrich). Visualization was achieved with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Sperm counts, motility and morphology

Caudal epididymal sperm were collected into Modified Whitten's medium. After 5 minutes, adequate dispersion of the sperm was determined visually, and an aliquot was diluted 1:20 in the same medium, which was placed on a pre-warmed counting chamber slide (100 μm in depth) (Cell Vision, Hopedale, MA, USA) and analyzed by computer-assisted semen analysis (CASA) on a Hamilton-Thorne IVOS V12.2L analyzer (Hamilton Thorne Research, Danvers, MA, USA). Five randomly chosen fields were analyzed. Motility (%) was recorded. For total sperm counts, two aliquots of sperm were diluted 1:200 in water for immobilization and put into a hemocytometer for counting. Afterwards, the average of sperm concentration was calculated. For analysis of sperm morphology, aliquots of caudal spermatozoa were prepared as above in pre-warmed M16 medium and diluted to 10^5/mL. A drop of paraformaldehyde was added to immobilize the sperm, 3 μl-aliquots were transferred into 12 μl MicroCell fixed-depth chambers (Conception Technologies, San Diego, CA) for visualization, and imaging was performed with differential interference contrast optics at × 40 magnification. At least 100 sperm were counted for each genotype, and both head and tail morphology were scored.

Fertility test

Adult male mice of three genotypes were caged with wild type female mice of two different genetic backgrounds (B6 and C57BL/6). The reproductive capacity evaluation was performed as described [11]. Male mice were housed with two female mice. Female mice were replaced weekly. When plugged female mice were found, they were removed and replaced immediately. Pregnancy was defined as plugged mice that gave birth to offspring. The number of total males and females, plugged females, litters, and total off-spring were counted to calculate frequency of copulatory plug (FCP), frequency of conception (FC) and average offspring amount (AOA).

Measurement of hormonal levels

Blood samples were taken from mice after they were killed. Samples were centrifuged for 15 minutes at 1000 × g, 4 Celsius degrees. Serum was removed and samples analyzed by ELISA kits (USCNLIFE, China) for testosterone and follicle-stimulating hormone (FSH). The optical density of the wells was determined with the ELISA reader SUNRISE (Tecan, Switzerland).

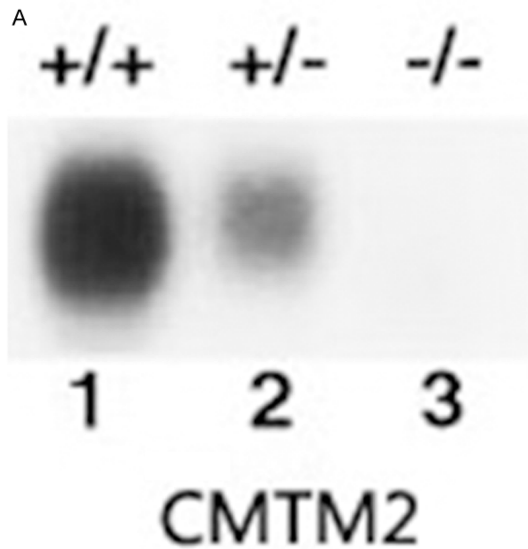
Statistics

Standard t-tests (Excel, Microsoft, Redmond, WA, USA) were used and standard error of means were calculated as appropriate. Statistical significance was defined as $P < 0.05$.

Results

Establishment of CMTM2-deficient mouse model

Interbreeding of heterozygous mice produced 36 *CMTM2*$^{+/+}$, 38 *CMTM2*$^{+/-}$ and 20 *CMTM2*$^{-/-}$ mice in F2 offspring. Among 255 mice from F1 to F8 generation, only 3 mice died, suggesting that CMTM2 deficiency didn't lead to embryonic lethality. The CMTM2-deficient mice grew to adulthood with no gross abnormalities and no increased lethality, and the adult CMTM2 null mice were normal in appearance. The body weight of adult mice with CMTM2 deficiency



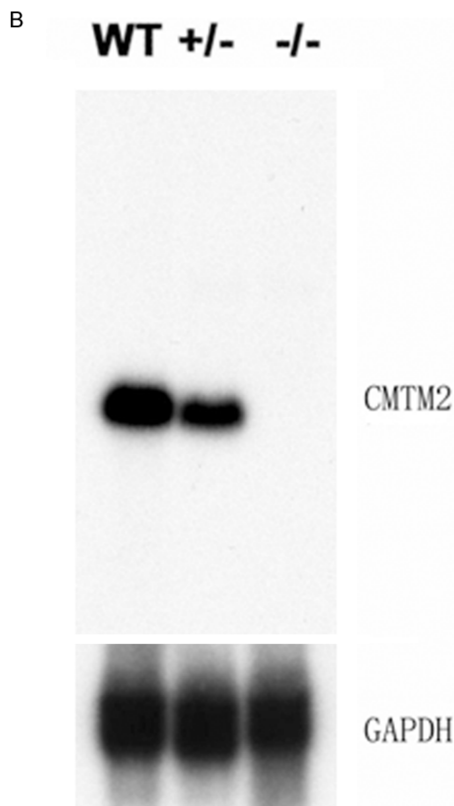


Figure 3. Analysis of testis gene expression in CMTM2 mutant mice. A. Northern blot analysis. The RNA from the mice whose genotype is shown above each lane was hybridized with a 32 P-labeled probe to detect the expression of the gene shown below each panel. The CMTM2 gene expression is abolished in *CMTM2*$^{-/-}$ animals. B. Western blot analysis Testes of *CMTM2*$^{+/+}$, *CMTM2*$^{+/-}$ and *CMTM2*$^{-/-}$ were tested for CMTM2 protein expression. There was a dramatic reduction in the expression of CMTM2 in mutant mice. GAPDH was used as the loading control.

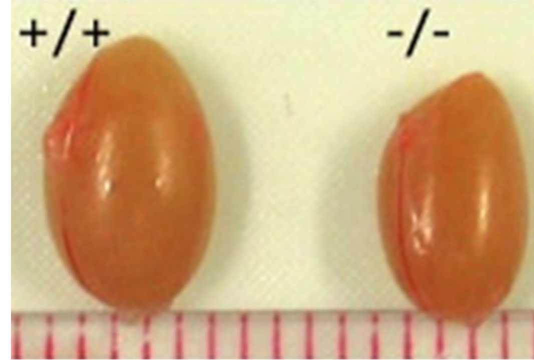


Figure 4. Comparison of the testis volume in CMTM2 mutant mice. The testes of *CMTM2*$^{-/-}$ mice are smaller than those of *CMTM2*$^{+/+}$.

was not significantly different from that of wild type mice. No obvious anatomical or behavioral abnormalities were observed. Consequently, targeted disruption of the CMTM2 gene in mice was successful (**Figure 3**). However, we noted that testes of *CMTM2*$^{-/-}$ were smaller than that of *CMTM2*$^{+/+}$ mice (**Figure 4**). The lower testis weight of *CMTM2*$^{-/-}$ male was not due to the loss of overall body weight, which was similar as indicated above (**Table 1**).

Sperm count, motility and morphology

The sperm count and motility of CMTM2 mutants and that of WT mice are significantly different. The *CMTM2*$^{-/-}$ mice produced virtually no sperm, and *CMTM2*$^{+/-}$ mice sperm count showed a significant decline (**Table 1**). The morphology changes are also significant, as there were more round sperms in the *CMTM2*$^{+/-}$ mice, and arrest of spermatogenesis could be observed in the *CMTM2*$^{-/-}$ mice, mostly round spermatids.

Serum hormone concentration

Serum testosterone and FSH concentrations in *CMTM2*$^{-/-}$ males (11.90±0.52 nmol/l and 5.33±0.12 mIU/ml, respectively), and *CMTM2*$^{+/-}$ (12.11±0.55 nmol/l and 5.39±0.15 mIU/ml, respectively) did not differentiate significantly from those in WT males (11.88±0.68 nmol/l and 5.34±0.19 mIU/ml, respectively).

Fertility outcome

Female CMTM2 null mice are fertile, indicating that CMTM2 is not required for female gameto-

Table 1. Reproductive physiology of CMTM2 mutant mice

Genotype	Age (week)	Testes weight (mg)	Sperm count (10^7/ml)	Motility (%)	Morphology
$CMTM2^{+/+}$	8-12	101.63±2.33	3.28±0.34	86.33±2.98	Normal
$CMTM2^{+/-}$	8-12	98.87±2.76	2.07±0.57	33.45±3.67*	Many round sperms
$CMTM2^{-/-}$	8-12	85.22±2.84*	0*	0*	0*

Values are mean ± SEM. Sperm counts and motility percentage was calculated by combining sperm from both caudal epididymides of mice. *P < 0.001.

Table 2. Fertility test of CMTM2 mutant mice

Male mice genotype	Female mice	Plugged mice	Pregnant mice	Offspring (M/F)	AOA	FCP (%)	FC (%)
$CMTM2^{+/+}$ (n=5)	WT (n=49)	38	31	249 (132:117)	8.0	77.6	63.3
$CMTM2^{+/-}$ (n=5)	WT (n=51)	34	13	103 (50:53)	7.9	66.7	25.5
$CMTM2^{-/-}$ (n=5)	WT (n=50)	36	0*	0*	0*	72.0	4*

M, male; F, female. Average offspring amount (AOA) was calculated as the total number of offspring divided by the number of females that gave birth to offspring. Frequency of copulatory plug (FCP) was calculated as the ratio of the number of plugged females to total number of females to which males with the same genotype had access. Frequency of conception (FC) was calculated as the ratio of the number of females that gave birth to offspring to total number of females. *P < 0.001.

genesis. In contrast, $CMTM2^{-/-}$ males are infertile. No significant differences were found among 3 groups in the ability of male mice of any genotype to plug wild type female mice, however, the $CMTM2^{-/-}$ mice failed to make female mice pregnant, and $CMTM2^{+/-}$ mice fertility was significantly reduced as the pregnant females and offspring were largely fewer than that of $CMTM2^{+/+}$ mice (**Table 2**).

Testes histology

The organized architecture of the seminiferous epithelium of the seminiferous tubules seen in $CMTM2^{+/+}$ mice was lost in $CMTM2^{-/-}$ mice (**Figure 5**). Contrary to the wild-type mice, the lumina of the seminiferous tubules in $CMTM2^{-/-}$ mice have a typically irregular contour or the lumen may even be obliterated. Furthermore, segments of $CMTM2^{-/-}$ mice seminiferous tubules have very few spermatogenic cells, only some round spermatids could be observed (**Table 3**). As their seminal fluid is devoid of mature spermatazoa, this indicated a complete block of spermatogenesis. The seminiferous epithelium of $CMTM2^{+/-}$ mice has reduced numbers of spermatogonia, resulting in reduced sperm production. There were no significant differences between the number and structure of Leydig cells and Sertoli cells between the 3 groups.

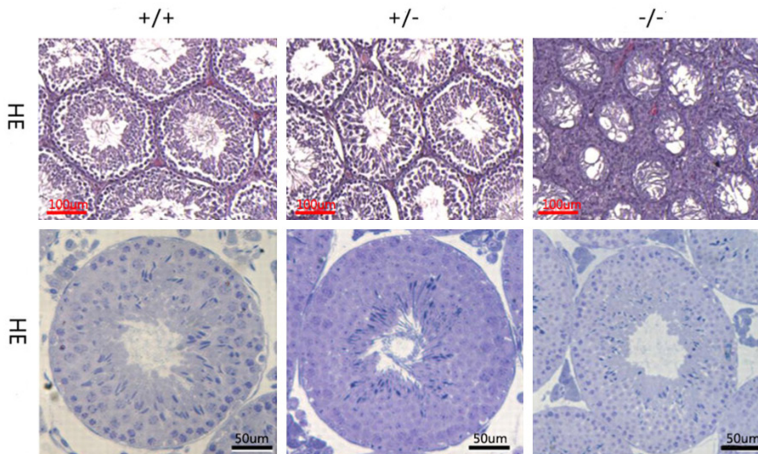


Figure 5. Testis histology in CMTM2 mutant mice. A. Histological staining of testis with hematoxylin and eosin (HE). Nearly complete germ cell loss is apparent in $CMTM2^{-/-}$ testis, whereas $CMTM2^{+/+}$, $CMTM2^{+/-}$ testes look normal. B. Spermatogonial depletion $CMTM2^{-/-}$ mice, $CMTM2^{+/-}$ mice look normal in comparison with $CMTM2^{+/+}$ animals, whereas a progressive germ cell loss is also could be observed.

Discussion

Our previous work has demonstrated that CMTM2 is associated with spermatogenesis in human [6, 9]. In the present study, we further investigated its role in CMTM2-deficient mouse models. Here, we reported that CMTM2 deficiency was associated with male infertility in mice.

Table 3. The number of cells in the seminiferous epithelium of seminiferous tubules in CMTM2 mutant mice

Genotype	SG	PSC	SSC	Spermatids	Sperms	Sertoli cells
CMTM2$^{+/+}$ (n=5)	39.11±2.98	67.92±1.03	2.96±0.73	139.22±7.81	40.84±5.92	9.21±1.29
CMTM2$^{+/-}$ (n=5)	37.73±4.55	63.34±6.01	2.29±0.74	128.18±8.17	29.28±3.44	9.45±1.78
CMTM2$^{-/-}$ (n=5)	14.22±5.01*	20.18±8.82*	0.00±0.00*	30.84±13.89*	0.00±0.00*	9.01±1.54

Values are mean ± SEM. SG-spermatogonia, PSC-primary spermatocytes, SSC-secondary spermatocytes. *$P < 0.001$.

The previous study showed that CMTM2 was localized to the endoplasmic reticulum near the Golgi apparatus [9]. As is known, during the post-meiotic germ cell development, spermatids undergo dramatic morphological transformations and structural modifications of the Golgi apparatus, centriolar-axonemal complex, mitochondria and endoplasmic reticulum [12]. In addition, CMTM2 contains MARVEL, a novel domain with a four transmembrane helix architecture that has been identified in proteins of the myelin and lymphocyte (MAL), physins, gyrins and occluding families. Their function could be related to cholesterol-rich membrane apposition events in a variety of cellular processes, such as biogenesis of vesicular transport carriers [13].

Fertility tests showed no significant differences in the ability of male mice of any genotype to plug wild type females. However, the *CMTM2*$^{-/-}$ mice produced no sperm, while the *CMTM2*$^{+/-}$ mice exhibited significantly reduced sperm count and motility which already affected fertility rate. In human, male infertility patients frequently have low sperm motility, and it is well known that sperm motility is a critical parameter that defines normal semen [14]. Men with impaired sperm motility have reduced fertilization possibility which may result in infertility [15]. Human beings with gene deficiency would be more vulnerable to pathological factors and lifestyles, including other genetic disorders, genital tract infections, environmental contamination and cigarette smoking [16]. It is acknowledged that accumulation of these mild defects could cause male infertility. In addition to multiple pathological factors, such as radiation and reactive oxygen species, genetic disorders can play a key role in sperm damage [17].

In contrast to females, male *CMTM2*$^{-/-}$ mice are sterile due to a complete block of spermatogenesis. CMTM2 deficiency is not lethal for development of spermatozoa within the epithelium of the seminiferous tubules in mice. The rare spermatozoa in *CMTM2*$^{-/-}$ mice are morphologically incomplete, and the spermatogenesis proceeds normally up until the transition from round to elongating spermatids. In wild-type mice, this transition is preceded by strong expression of CMTM2 in the nuclei of round spermatids. Simultaneously, CASA revealed differences in sperm motility between CMTM2-deficient mice and wild type mice. Given that CMTM2 is a novel protein that has been cloned recently, its biological function remains unclear [18, 19]. It is proposed that the arrest in spermiogenesis at the transition from round to elongating spermatids may be explained by the absence of expression of specific haploid genes, though we did not examine the expression of the critical activator protein CREM [20, 21] as well as that of many other well-characterized testis-specific genes. The expression of these genes normally begins in step 5-7 round spermatids, and their mRNAs persist in elongating spermatids [22, 23]. The onset of their transcription, therefore, coincides with a peak of CMTM2 expression, and although we cannot exclude an indirect effect, this suggests that their expression may be directly controlled by CMTM2. These genes have not yet been fully characterized, and their precise functions are not known. Nevertheless, in the absence of their expression in CMTM2 null mice spermiogenesis is arrested, suggesting that they are essential for the maturation of elongating spermatids.

There are several limitations for this article: first of all, as this is only an early phase observation study on CMTM2 in mice, we did not conduct the time-based dynamic observational study on CMTM2 null mice; secondly, we did not conduct the apoptosis study to elucidate whether excessive apoptosis is involved in the reduced spermatogenesis, and we also did not conduct many other studies, like electron microscopy, *in situ* hybridization, immmunofluo-

rescence microscopy. We are going to provide these above mentioned study results in future study, and will try to elucidate the mechanisms of CMTM2 involvement in spermatogenesis.

However, the molecular and cellular mechanism of CMTM2 remains elusive and needs further investigation. In conclusion, we successfully generated mouse model with CMTM2 deficiency, and confirmed the reproductive phenotype, fertility status and testis changes of the CMTM2 null mice. Furthermore, we believed the CMTM2 is involved in the post-meiotic phase spermatogenesis and apoptosis. Here we report that CMTM2 is one of the genes likely involved in male infertility, and further study is required.

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Disclosure of conflict of interest

None.

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