Localization and Characterization of Rat Transmembrane Protein 225 Specifically Expressed in Testis

Shirui Yang,^{1,*} Weiping Wang,^{1,*} Chen Lei,¹ Qingmei Liu,¹ Fengqin Xu,¹ Xiaowei Xing,² Hao Chen,¹ Jiajia Liu,¹ Shiliang Wu,¹ and Minghua Wang¹

Testis is the one and only location of spermatogenesis and sexual hormone production. Spermatogenesis is a complicated physiological process regulated by many genes specifically and differentially expressed in the testis. In this study, Transmembrane Protein 225 (TMEM225), which is specifically expressed in rat testis, has been identified. *TMEM225* was cloned from the testis cDNA library and was mapped to chromosome 8q22 by browsing the University of California Santa Cruz genomic database. It contains an open reading frame with a length of 696bp, encoding a protein with four putative transmembrane helices. *TMEM225* mRNA expression was evaluated by reverse transcription–polymerase chain reaction and *in situ* hybridization. In addition, the subcellular location of *TMEM225* was evaluated. The results obtained highlighted age-related specific expression of *TMEM225* in testis, specifically during the adult period after age of 13 months. *In situ* hybridization analysis indicated that *TMEM225* mRNA was mainly expressed in spermatocyte cells and round spermatids. Green fluorescence protein localization analysis showed that rat *TMEM225* mainly surrounded the nuclear membrane, with a minority distribution in the cytoplasm, and the distribution of *TMEM225* was affected by the deletion of N-terminal transmembrane domain. As the expression phase is not related to the first wave of spermatozoon development, our data presented here suggest that *TMEM225* may play an important role in sperm degeneration but not in spermatogenesis.

Introduction

A TRANSMEMBRANE PROTEIN is a protein that spans the entire biological membrane one or a few times. To date, hundreds of transmembrane proteins have been identified (Aruga and Mikoshiba, 2003; Haugstetter *et al.*, 2005; Schweneker *et al.*, 2005). Tetraspans are a group of membrane proteins with four transmembrane domains, which span the membrane four times. Tetraspans transmembrane family members, also known as tetraspanin superfamily, have various effects on cell proliferation, motility, and adhesion in different cells (Morita *et al.*, 1999; Christophe-Hobertus *et al.*, 2001; Hulett *et al.*, 2001; Mazzocca *et al.*, 2002; Schweneker *et al.*, 2005). For example, TM4SF protein was found to form massive tetraspanin-enriched microdomains and functions collaboratively with integrins in cell adhesion and motility (Mazzocca *et al.*, 2002). Tight junctions are very important for barrier function in epithelial and endothelial cells, and four-transmembrane helices protein Claudin11 was found to be a tight junction protein at the human blood–testis barrier (Fink *et al.*, 2009).

Spermatogenesis and sexual hormone production only exist in testis. Spermatogenesis is a complicated physiological

process including cell division (Eberhart and Wasserman, 1995), differentiation, and interactions between cells in the seminiferous tubule (Takenaka *et al.*, 2008). A large number of transmembrane proteins have been reported to be involved in spermatogenesis (Endo *et al.*, 1996; Cheng *et al.*, 2003; Hyenne *et al.*, 2007; Sze *et al.*, 2008). Recently, many testis-expressed transmembrane proteins have been discovered. Testis-specific expression protein TETM4 has four strong transmembrane helices and was suggested to be associated with receptor complexes on the surface of specific cells in the testis and participated in signaling events (Hulett *et al.*, 2001). Sdmg1, a conserved transmembrane protein, was found to be associated with germ cell sex determination and germline–soma interactions in mice (Best *et al.*, 2008). Degenerative spermatocyte, a novel gene encoding a transmembrane protein, may be required for the initiation of meiosis in spermatogenesis (Endo *et al.*, 1996). Membrane protein Tmem184a expressed exclusively within the Sertoli cells of the developing testis cords was reported to mediate sex-specific signaling events during Sertoli cell differentiation (Svingen *et al.*, 2007).

Despite the fact that the complete nucleotide sequence of the human genome is known, many genes encoding membrane proteins were continuously discovered. So far, some

¹Department of Biochemistry and Molecular Biology, Medical School, Soochow University, Suzhou, China.

²The Center for Experimental Medicine Research, Third Hospital of Central South University, Changsha, China.

*These two authors contributed equally to this work.

membrane proteins remain unidentified. Using bioinformatics and an experiment validation method, we have identified a novel transmembrane protein TMEM225, which is specifically expressed in rat testis. The TMEM225 protein is composed of four transmembrane helices. In the present study, reverse transcription-polymerase chain reaction (RT-PCR) *in situ* hybridization analysis, and green fluorescence protein (GFP) subcellular localization were employed to delineate the function of TMEM225. The results obtained highlighted age-related specific expression of TMEM225 in testis, specifically during the adult period after age of 13 months. *In situ* hybridization analysis indicated that TMEM225 mRNA was mainly expressed in spermatocyte cells and round spermatids. GFP localization analysis showed that rat TMEM225 mainly surrounded the nuclear membrane, with a minority distribution in the cytoplasm, and the distribution of TMEM225 was affected by the deletion of N-terminal transmembrane domain. The data presented here suggest that TMEM225 may play an important role in sperm degeneration but not in spermatogenesis.

Materials and Methods

Cloning of rat gene TMEM225

BLAST search at University of California Santa Cruz (UCSC) genome browser (<http://genome.ucsc.edu>) with the reported mouse unnamed protein (GenBank accession number: BAB24641) allowed us to identify a 2.6-kb homologous region in rat genome, in which no known protein was identified. According to the genomic DNA sequence in this region, primers (sense: 5'-GAT GGT AGT CTC TGG TAGA-3'; anti-sense: 5'-TGC AGA CCA TCA AGT CAC AG-3') were designed and synthesized (Biocolour BioTech). PCR was performed with adult rat testis cDNA as template. The PCR conditions were as follows: 94°C for 5 min, 35 cycles of 20 s at 94°C, 30 s at 56.5°C, and 50 s at 72°C, followed by a final extension of 5 min at 72°C. The PCR product was subjected to T-A cloning and was sequenced on an ABI PRISM sequencer.

In silico analysis

To determine the mapping information, the cDNA sequence of TMEM225 was applied for genomic searching at <http://genome.ucsc.edu>. BLASTP tool (www.ncbi.nlm.nih.gov) was applied for identifying the orthologs of TMEM225 in different species. The SMART tool was used for domain searching (<http://smart.embl-heidelberg.de>), and the Vector NTI package (Informax) for protein molecular weight prediction. PSORT (<http://psort.nibb.ac.jp>) was used for subcellular localization prediction. The HMMTOP server (www.enzim.hu/hmmtop) was used for the prediction of the transmembrane helices and topology. Vector NTI software was used for protein sequence alignment analysis. The GenBank accession numbers used for analysis were NP_001019530 (rat), NP_001013765 (human), XP_001137001 (chimpanzee), XP_001108697 (monkey), BAB24641 (mouse), XP_852701 (dog), NP_001073753 (cow), and XP_001501682 (horse).

Expression pattern analysis of TMEM225

The expression pattern of TMEM225 was determined by RT-PCR (Huang *et al.*, 2004). Total RNA was extracted from

eight different tissues (lung, liver, ovary, spleen, testis, heart, kidney, and skeletal muscle) with a single-step isolation method using TRIzol reagent according to the manufacturer's instructions. The cDNA was synthesized using 2 µg of total RNA, Superscript II reverse transcriptase (Gibco BRL), and Oligo (dT) 15 (Promega) as per the manufacturer's protocols. The 423-bp product was amplified on an FS-918 DNA Amplifier (Shanghai Fusheng Institute of Biotechnology) using the primer pair TMEM225-F (5'-ATA AAG TTA CCC ACA GTC C-3') and TMEM225-R (5'-TCA TTG CTT TGC TGC TAC-3'). The PCR conditions were as follows: 94°C for 2 min, 35 cycles of 30 s at 94°C, 30 s at 52°C, and 40 s at 72°C, followed by a final extension of 5 min at 72°C. To determine age-related expression of TMEM225 in rat testis, RNA were also extracted from rat testis at different ages (1 day, 15 days, 35 days, 65 days, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 12 months, 13 months, 14 months, 15 months, and 18 months). Using the primer pair of TMEM225-RT-A (5'-ATG ATG CGC ATT CCA AAC AGA A-3') and TMEM225-RT-B (5'-TCA CAG AGC CCA GGT CAC ACG A-3'), PCR product size was 696 bp. The PCR conditions were as follows: 94°C for 2 min, 35 cycles of 30 s at 94°C, 30 s at 53°C, 50 s at 72°C, followed by a final extension of 5 min at 72°C. The expression of the β-actin was analyzed as a control (sense primer: 5'-GGC CCC TCT GAA CCCT AAG-3'; anti-sense primer: 5'-TGC CAC AGG ATT CCA TAC CC-3'). The PCR conditions were the same as for TMEM225 except that the annealing temperature was changed to 58°C. PCR product size was 500 bp.

In situ hybridization

The mRNA expression of TMEM225 in rat testis was determined by *in situ* hybridization technique (Xing *et al.*, 2004). Sprague-Dawley rats (6 and 14 months) were sacrificed and rat testis tissues were fixed in ice-cold 4% paraformaldehyde for 2 h and then soaked in 40% sucrose overnight. The tissues were frozen at -20°C and 30-µm sections were cut. TMEM225 *in situ* hybridization kit was purchased from Boster Bioengineering. As longer probes were difficult to penetrate the cell, short oligonucleotide probes were used in this study. To strengthen the signals, three probes were used. The sequences of oligonucleotide probes were as follows: (1) 5'-CCA CAG TCC ATG GCT GGG ATG CTG TCC TCC TTT-3'; (2) 5'-GCA GTT CAC CTA CAT GAT TTC TCA AAA TAA GTG TG-3'; (3) 5'-AAC AGA TGT GCG TGC ATG AAA TTC TGT GTA CCC CA-3'. The probes were labeled with digoxigenin (Boster Bioengineering). Prehybridization was performed for 3 h. Subsequently, hybridization was performed in a solution that contained digoxigenin-labeled TMEM225 probes. The control sections were treated with the solution without TMEM225 probes. Posthybridization washes were performed at 37°C by incubation in 2 × SSC twice (each for 5 min), in 0.5 × SSC for 15 min, and in 0.2 × SSC twice (each for 15 min). Sections were incubated in blocking buffer for 30 min at 37°C and then incubated with biotin-labeled antidigoxigenin antibody at 37°C for 2 h. Slides were washed in 0.5 M PBS (pH 7.2–7.6) four times, each for 5 min. Sections were incubated in SABC at 37°C for 20 min. Slides were washed in 0.5 M PBS (pH 7.2–7.6) three times, each for 5 min. After that, sections were incubated in biotined peroxidase at 37°C for 20 min. Slides were washed in

0.5 M PBS (pH 7.2–7.6) four times, each for 5 min. The specific signals were visualized by incubation in diaminobenzidine buffer. Water was used to end the reactions. Sections were redyed with hematoxylin and photographs were taken. Negative controls were subjected to the same conditions as above.

Analysis of subcellular localization

The PCR product of the complete *TMEM225* open reading frame (ORF) with *Xho*I and *Eco*RI restriction sites was subcloned into the *Xho*I/*Eco*RI sites of pEGFP-C1 (Clontech) and the recombinant green fluorescence expression vector pEGFP-TMEM225 was produced. Restriction sites had been introduced by the forward (5'-ATC TCG AGC AAT GAT GCG CAT TCC-3') and reverse (5'-ATG AAT TCA GTC ACA GAG CCC AGG-3') primers. The N-terminal 35 amino acids-deleted mutant pEGFP-D35-TMEM225 was also constructed by PCR using the forward (5'-ACC TCG AGA ATT GAT TCC CAA AGTG-3') and reverse (5'-TGA ATT CAG TCA CAG AGC CCA GG-3') primers. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco BRL) and 1% penicillin/streptomycin at 37°C in a 5% humidified CO₂ atmosphere. Cells were cultured directly on glass coverslips in six-well plates at a density of approximately 2×10^5 cells per well and incubated for 24 h. The incubation was continued for another 3 h in the presence of the Superfect Reagent (Qiagen), 1.7 µg pEGFP-TMEM225 or pEGFP-D35-TMEM225, and pEGFP-C1 green fluorescence protein expression vector as a control. The medium was replaced by

10% charcoal-treated fetal calf serum (DCC; Hyclone). The cells were incubated for 48 h and then washed three times with PBS (pH 7.4). The HeLa cells were fixed in 3% paraformaldehyde for 20 min and then permeabilized with 0.5% Triton X-100 in PBS for 5 min. After rinsing with PBS, DAPI (4',6-diamidino-2-phenylindole; 2.5 µg/ml; Invitrogen) in PBS was used to stain the nucleus for 30 min and visualized with a microscope equipped with fluorescence optics (Leica DM R2).

Results

Rat TMEM225 gene and its genomic organization

Mouse unnamed protein (GenBank accession number: BAB24641) was found to be transcribed from the testis, so the rat testis cDNA was used as a template for PCR amplification. A single band with a size of about 696 bp was excised from the agarose gel, cloned into pMD18-T vector (TaKaRa), and then subjected to sequencing. The sequence was submitted to GenBank with the accession no. AY634367 and with the name *PMP22CD*, but its homologs in human and mouse were renamed as *TMEM225* because it was not related to the claudin family. So we also used the gene name *TMEM225* for rat *PMP22CD*. *TMEM225* cDNA encodes a protein with 231 amino acids (Fig. 1). The molecular weight and isoelectric point are predicted to be 26.7 kDa and 9.45, respectively, using Vector NTI software. It is composed of four exons and three introns (Table 1), the open reading frame is from 119 to 814 nucleotides, the ATG start codon

1	C	TCT	GTC	AAC	ATC	TCT	GCA	GTC	AGT	GAA	CAT	CCC	GAG	TTC	ATC	CTG	CAT	CTT	CTG	ACT
59	AAT	CAA	ATC	AAG	GAC	ATA	TAA	AAC	ATT	GAT	GGT	AGT	CTC	TGG	TAG —AAA	TTT	TTA	TCC	ACA	
	M	M	R	I	P	N	R	S	I	Q	A	A	N	I	F	F	S	S	G	A
119	ATG	ATG	CGC	ATT	CCA	AAC	AGA	AGT	ATC	CAG	GCT	GCC	AAC	ATA	TTC	TTC	TCC	TCT	GGG	GCC
	I	L	L	L	I	A	G	L	I	M	E	N	W	V	E	L	I	P	K	V
179	ATA	CTT	CTG	TTG	ATA	GCA	GGA	CTC	ATC	ATG	GAA	AAC	TGG	GTG	GAA	TTG	ATT	CCC	AAA	GTG
	R	K	D	K	V	T	H	S	P	W	L	G	C	C	P	P	F	W	P	E
239	AGA	AAA	GAT	AAA	GTT	ACC	CAC	AGT	CCA	TGG	CTG	GGA	TGC	TGT	CCT	CCT	TTT	TGG	CCT	GAA
	E	S	L	E	A	I	R	R	M	M	M	M	S	L	N	I	S	I	Y	L
299	GAG	AGC	CTG	GAG	GCA	ATC	AGA	CGG	ATG	ATG	ATG	ATG	AGT	CTC	AAC	ATT	TCC	ATC	TAT	CTA
	N	L	I	I	G	L	Q	F	T	Y	M	I	S	Q	N	K	C	V	H	L
359	AAC	TTG	ATC	ATA	GGT	CTG	CAG	TTC	ACC	TAC	ATG	ATT	TCT	CAA	AAT	AAG	TGT	GTG	CAT	CTC
	L	I	G	F	L	S	F	F	T	G	C	L	L	F	Y	A	I	I	V	Y
419	CTT	ATT	GGC	TTC	CTG	AGT	TTC	TTC	ACA	GGT	TGC	CTT	TTG	TTC	TAT	GCA	ATC	ATT	GTG	TAT
	H	H	K	L	N	K	G	Q	Y	V	Y	F	V	N	Y	K	T	K	W	I
479	CAT	CAC	AAG	CTA	AAT	AAA	GGT	CAG	TAT	GTG	TAC	TTC	GTT	AAT	TAC	AAG	ACC	AAA	TGG	ATA
	V	F	T	I	Y	L	T	I	A	L	F	L	T	C	G	I	F	S	F	I
539	GTT	TTC	ACC	ATC	TAC	TTA	ACC	ATC	GCC	CTC	TTT	CTT	ACC	TGT	GGT	ATC	TTC	AGT	TTC	ATA
	Q	C	T	N	R	C	A	C	M	K	F	C	V	P	H	T	E	S	S	S
599	CAG	TGC	ACA	AAC	AGA	TGT	GCG	TGC	ATG	AAA	TTC	TGT	GTA	CCC	CAC	ACA	GAA	AGT	AGC	AGC
	K	A	M	T	Q	N	T	I	Q	V	I	S	L	P	P	R	S	E	M	P
659	AAA	GCA	ATG	ACT	CAG	AAT	ACA	ATC	CAA	GTT	ATT	TCA	CTT	CCT	CCA	CGA	TCG	GAA	ATG	CCT
	R	S	I	V	H	M	H	S	D	M	P	G	K	E	G	S	I	S	K	P
719	CGA	AGT	ATC	GTC	CAT	ATG	CAC	TCT	GAT	ATG	CCA	GGC	AAG	GAG	GGT	TCC	ATA	TCC	AAA	CCA
	H	L	Q	S	R	R	V	T	W	A	L	*								
779	CAT	CTC	CAA	TCA	CGT	CGT	GTG	ACC	TGG	GCT	CTG	TGA	CTT	GAT	GGT	CTG	CAC	CTT	TCA	TAC
839	TGT	CAT	CCT	GTC	ATT	GCT	ATG	GAA	TTA	ACT	GTA	TGT	TTC	CAA	TCG	ATC	ATC	TGT	ATG	GCA
899	ATG	GAA	AGA	TTC																

FIG. 1. Nucleotide sequence and deduced protein sequence of the rat *TMEM225* gene. *TMEM225* encodes a polypeptide of 231 amino acids; amino acids are identified by their one-letter code; nucleotides are numbered at the left side of each line. *TMEM225*, transmembrane 225; *, the termination codon TGA; TAG, in-frame stop codon.

TABLE 1. GENOMIC STRUCTURE OF TRANSMEMBRANE 225 GENE

3' Splice acceptor	Exon	Size (bp)	5' Splice donor	Intron	Size (bp)
cDNA end CGACGCTCCG	1	299	TGGCCTGAAGgtcagaacta	1	684
ttccaacagAGAGCCTGGA	2	147	TTCTTCACAGgtagcttct	2	270
tgaacactagGTTGCCTTTT	3	135	CTTACCTGTGgtaagtatcc	3	695
ctgtccctagGTATCTTCAG	4	329	GGAAAGATTC aaaaaaaaaa	PolyA	

Exon sizes are given in bp and the exonic and intronic sequences at the splice junction are shown in capital and lowercase letters, respectively. The exon-intron splicing signals gt and ag are in bold.

(nucleotides 119–121) is preceded by an in-frame stop codon TAG, and there is not any typical polyadenylation signal.

In silico analysis of TMEM225 gene

TMEM225 was mapped to rat chromosome 8q22 by browsing the UCSC genomic database (<http://genome.ucsc.edu>). Another interesting finding was that the gene TMEM225 and 41 olfactory receptor genes (Olr1301 to Olr1341) are located closely to each other and cluster together in the same chromosome loci rat 8q22. The use of putative transmembrane domain prediction software (HMMTOP) revealed that the TMEM225 protein is composed of four alpha-helical transmembrane domains (TM-1, TM-2, TM-3, and TM-4) of 20, 23, 20, and 20 amino acids, respectively, N- and C-terminal cytoplasmic domains of 10 and 71 amino acids, respectively, two intracellular loops of 39 and 20 amino acids, and a short extracellular loop of eight amino acids (Fig. 2). The HMMTOP analysis also revealed that the N-terminus is outside of the membrane, the first loop of TMEM225 is longer than the second one, and the TMEM225 contains a very short N-terminus tail, but a more than 71-residue-long C-terminus. A BLASTP search of published protein databases of NCBI for sequences similar to that of TMEM225 indicated that it had orthologs in human, chimpanzee, monkey, dog, mouse, cow, and horse. Multiple alignment analysis was made by Vector NTI. Figure 3 showed that TMEM225 was highly conservative in different species: there were six serine residues and seven leucine residues highly conserved in all the species, and five amino acids block PRSIV and five amino acids block VTWAL were highly conserved in its C-terminus. Rat TMEM225 shares

homology with the orthologs in human (43% identity and 64% similarity), chimpanzee (44% identity and 64% similarity), monkey (43% identity and 63% similarity), dog (45% identity and 65% similarity), mouse (83% identity and 91% similarity), cow (41% identity and 61% similarity), and horse (40% identity and 62% similarity).

Age-dependent specific expression of TMEM225 in rat testis

RT-PCR was used to determine the expression pattern of the gene. To investigate the tissue distribution of rat TMEM225, RNA from eight different tissues including lung, liver, heart, testis, skeletal muscle, kidney, and ovary were extracted and reverse transcribed into cDNA and then were analyzed by PCR. The tissue distribution pattern of TMEM225 mRNA was shown in Figure 4. Among the tissues analyzed, TMEM225 expression was detected only in the testis. It suggested that TMEM225 was specifically expressed in testis. To determine whether the expression of TMEM225 was age dependent, testis from rats of different ages were analyzed by RT-PCR. Figure 5 shows that TMEM225 began to be expressed in adult rat testis around 13 months of age.

Analysis of TMEM225 mRNA expression in rat testis by in situ hybridization

To further investigate the cell distribution of TMEM225 mRNA in testis, testis from rats aged 6 and 14 months were analyzed by *in situ* hybridization. In Figure 6, the presence of TMEM225 mRNA is indicated by brown staining of cytoplasm of spermatocyte cells and round spermatids, whereas no obvi-

TMEM225	MMRIPNRSIQ	AANIFFSSGA	ILLIAGLIM	ENWVELIPKV	RDKVTHSPW	50
Pred	OOOOOOOOO	HHHHHHHHH	HHHHHHHHH	iiiiiiiiiii	iiiiiiIIIII	
TMEM225	LGCCPPFWPE	ESLEAIRRM	MMSLNISIYL	NLIIGLQFTY	MISQNKCVHL	100
Pred	IIIIIIIIIII	IIIIIIiiih	HHHHHHHHH	HHHHHHHHH	HHOOOOOOO	
TMEM225	LIGFLSFFTG	CLLFYAIIVY	HKLNKGQYV	YFVNYKTKWI	VFTIYLTIAL	150
Pred	HHHHHHHHH	HHHHHHHHH	iiiiiiiiiii	iiiiiiiiiii	HHHHHHHHH	
TMEM225	FLTCGIFSFI	QCTNRCACMK	FCVPHTESSS	KAMTQNTIQV	ISLPPRSEMP	200
Pred	HHHHHHHHH	OOOOOOOOO	OOOOOOOOO	OOOOOOOOO	OOOOOOOOO	
TMEM225	RSIVHMHSDM	PGKEGSISKP	HLQSRRTWA	L	231	
Pred	OOOOOOOOO	OOOOOOOOO	OOOOOOOOO	O		

FIG. 2. Predicted structure of TMEM225 in the membrane. The structure was drawn based on the prediction of HMMTOP. TMEM225 shows four transmembrane domains with both the N- and C-termini extracellularly and a long C-terminal domain.

		1	60
Rat	(1)	MMHPIPNRSIQAAANIFFSSGAILLIITAGLIMENWVELIPKVRKDKVTHSPULGCCPPFWPE	
Human	(1)	MVHVSNNRSIQGMNIFSSWAVVLMVHGITLTKWVELISEDERAKNNHSPWMMCCPALWPE	
Chimpanzee	(1)	MVHVSNNRSIQGMNIFSSWAVVLMVHGITLTKWVELISEDERAKNNHSPWMMCCPALWPE	
Monkey	(1)	MVHVSNNRSIQGMNIFSSWAVVLMVHGITLTKWVELISEDEGIKINHSPWMTCCPAWPE	
Mouse	(1)	MMHPIPNRSIQAAANIFFSSGAILLIIVGLIMEDWVELIPKVRKDKTTHSPULGCCPPFWPE	
Dog	(1)	MAKVSRLRGIQATNMFFSSWALIFLTGLILREEWVELTLETKKNTISHSPWICCT-TLWPE	
Cow	(1)	-----HFFSSWTLVFLAVGIIIEEWAEKLGPKPTIITHSPWICCT-PLWPS	
Horse	(1)	MVHISTRNVQATNMLFSSWALVFLAVGIIIEEWVELKFETKNDTISHSPWICCT-SVWPE	
		61	120
Rat	(61)	ESLEAIRMMMSLNIS IYLNLIIGLOFTYMI SONKCVHLLIGFLSFFTGCLLFYAIIVY	
Human	(61)	DDLKVVRIIMTSSLGLSFLNLILGHKFTYLIPQNKYIQLFTTILSFFSGISLLWALILY	
Chimpanzee	(61)	DDLKVVRIIMTSSLGLSFLNLILGHKFTYLIPQNKYIQLFTTILGFFSGIFLLWALILY	
Monkey	(61)	DDLQVVRIIMMS ILGLSFLNLILGHKFTYLIPENKSLQLFATILSFFSGILLWALMLY	
Mouse	(61)	ESLEVVRIMRMTLNIS IYLNLIIGLOFTYMI SONKCVHLLIGFLSFFTGCLLFYAIIVY	
Dog	(60)	DGLEVVRIIMMILCLSLSFVHNLFLGLEFTYFIPQTKYVFFITVFLSFFTGCILLCALILY	
Cow	(47)	DGLEVIRNLIIVLSLSFMHNLFLGLEFTYFIPQTKYILIMTACLAFLTGILLGALLLY	
Horse	(60)	DNLKVVRIIMIVLSLSFFTNNFLGLEFTYLLPQSKLVLFVNVFLSFLSGILLCALLY	
		121	180
Rat	(121)	HHKLNKGQYVYFVNYKTKWIVFTIYLTIALFLTCGIFSF IQCTNRCACMKFCVPHTESS	
Human	(121)	HNKLKQGQSMHFSNYRITWIMYTAYLNVFVFLSVCGILSLECKLSTSSCTCLNIHKS DNE	
Chimpanzee	(121)	HNKLKQGQSMHFSNYRITWIMYTAYLNVFVFLSVCGILSLECKLSTSSCTCLNIHKS DNE	
Monkey	(121)	HNKLKQGQSVHFSSYRITWIMNTAYLNVFVFLSVCGILSLECKLSTGCTCLNIHTSDTE	
Mouse	(121)	HHKLNKGQYVYFVNYKTKWIAFTVYLTIALFLTCGIFCF IQSTNRCECMKFCIPHTESKS	
Dog	(120)	QLKLKQGQSVYVSTYKITWIIIFTAYLSVSFFMASGILSLECKKSTACACATLIHTPER	
Cow	(107)	HHMLRQGESVYVSSYKISWIIIFTAYLNVLFLFISGFLSLLQYKQPIDGSG--SLIPRSAR	
Horse	(120)	RRNLKQGQSVYVASYRITWIIIFTAYLNVFVFFIVSGILSVLQHKQINCCGSLTILHESER	
		181	235
Rat	(181)	KA---MTONTICVISLPPRSEMPRSIVHMSDMPGKEGSIKPHLOSRRVTWAL	
Human	(181)	CK---ESENSIEDISLPECTAMPERSIVRAHTVNS-----LNKKVQTRRVTWAL	
Chimpanzee	(181)	CK---ESENSIEDISSPERTAMPERSIVRAHTVNS-----LNKKVQTRRVTWAL	
Monkey	(181)	CK---ESENSIEGTSLPEHAAKPRISIVRAHTVNS--KEDILNKQVQTRRVTWAL	
Mouse	(181)	QE---MIPSTIEVSLPPRCAMPERSIVHVSHTS-KDGSINRPHTQARRVTWAL	
Dog	(180)	ESEDEESESSESVKIVSLPENAAAPRSIVHTR-----E-GSPNRPQLOTRRVTWAL	
Cow	(165)	KSQVMEQHGVSIVKVSLLPAGTAMPERSIVRLHSAHMKE-DSPERLNIAQARRVTWAL	
Horse	(180)	EIQDIQSESSVFTSLPARTAMPERSIVRVHSTHTGE-DSPNKSQIQTRRVTWAL	

FIG. 3. The sequence alignment of rat TMEM225 protein and its orthologs. The sequences used for alignment include those of rat (NP_001019530), human (NP_001013765), chimpanzee (XP_001137001), monkey (XP_001108697), mouse (BAB24641), dog (XP_852701), cow (NP_001073753), and horse (XP_001501682).

FIG. 4. Expression pattern of TMEM225 in eight rat tissues.

ous positive signals were detected in negative control sections. The expression of TMEM225 mRNA was only detected in rats aged 14 months but not in 6-month-old rats (data not shown).

TMEM225 protein expression and its subcellular location

To investigate the subcellular location of our interested protein, the PSORT prediction tool and signalP3.0 server were used. The subcellular location prediction results showed that TMEM225 was localized in the cytoplasm, and there was a high potentiality of localization in the endoplasmic reticulum.

FIG. 5. Analysis of age-dependent expression of TMEM225 in rat testis by RT-PCR. M: 100-bp DNA ladder; lanes 1-17: 18-month-, 15-month-, 14-month-, 13-month-, 12-month-, 11-month-, 10-month-, 9-month-, 8-month-, 7-month-, 6-month-, 5-month-, 4-month-, 65-day-, 35-day-, 15-day-, 1-day-old rat, respectively. The expression of β-actin was analyzed as a control.

FIG. 6. *In situ* hybridization of *TMEM225* mRNA in 14-month-old rat testis tissue. The *TMEM225* mRNA was detected in spermatocyte cells and round spermatids of 14-month-old rat testis, using oligonucleotide probes. The presence of *TMEM225* mRNA was revealed by brown staining (**A**, **B**). No significant brown signals were detected in the seminiferous tubules of controls (**C**, **D**). The cell nucleus was stained purple with hematoxylin. (**A**) Fourteen-month-old rat testis under low-power lens; (**B**) 14-month-old rat testis under high-power lens; (**C**, **D**) negative control under low- and high-power lens, respectively. Color images available online at www.liebertonline.com/dna.

FIG. 7. Subcellular localization of *TMEM225* protein in HeLa cells. (**A–C**) Cells transfected with pEGFP-C1. (**D–F**) Cells transfected with pEGFP-*TMEM225*. (**G–I**) Cells transfected with pEGFP-D35-*TMEM225*. (**A**, **D**, **G**) The nucleus of cells were stained with DAPI. (**C**) Colocalization of **A** and **B**. (**F**) Colocalization of **D** and **E**. (**I**) Colocalization of **G** and **H**. Color images available online at www.liebertonline.com/dna.

The signal sequence prediction results displayed that the 1–35 amino acids have the high possibility to belong to the signal anchor (data not shown). To verify the subcellular location and the function of N-terminal residues in the location of our interested protein, wild pEGFP-TMEM225 and truncated pEGFP-D35-TMEM225 were transfected into HeLa cells. As shown in Figure 7, when pEGFP-TMEM225 were overexpressed in HeLa cells, we found that the strongest green fluorescence concentrated around the nuclear membrane, and we also observed granular green fluorescence scattering in the cytoplasm and did not detect any fluorescence signal in the cell membrane. In contrast, we observed that the green fluorescence was distributed throughout the cell in the control group, which was transfected with pEGFP-C1. The green fluorescence did not concentrate around the nuclear membrane anymore, when the mutant pEGFP-D35-TMEM225 overexpressed in HeLa cells, and the fluorescence was dispersed in the cytoplasm.

Discussion

In the present study, we report the isolation and characterization of a novel rat gene *TMEM225*, which is mapped to chromosome 8q22. It contains an ORF with a length of 696bp, encoding a protein with four putative transmembrane helices, which are likely to adopt a membrane topology with both the N- and C-termini extracellularly. On the basis of the transmembrane domain prediction, the TMEM225 protein can be divided into the following domains: four transmembrane domains (TM-1, TM-2, TM-3, and TM-4) of 20, 23, 20, and 20 amino acids, respectively, N- and C-terminal cytoplasmic domains of 10 and 71 amino acids, respectively, two intracellular loops of 39 and 20 amino acids, and a short extracellular loop of 8 amino acids (Fig. 2).

Previous studies have shown that most tetra-transmembrane proteins locate at the cell membrane or endoplasmic membrane (Namiki *et al.*, 1988; Christophe-Hobertus *et al.*, 2001; Haugstetter *et al.*, 2005; Schweneker *et al.*, 2005). We also determined the localization of TMEM225 in the cell; however, it is not located at the cell membrane. On the contrary, TMEM225 protein is granularly scattered in the cytoplasm and is mainly concentrated around the nuclear membrane (Fig. 7D–F). As the outer nuclear membrane is continuous with the membrane system of the endoplasmic reticulum, TMEM225 protein localization is similar to the proteins targeting into the endoplasmic reticulum. The cellular distribution of TMEM225 was significantly changed by the deletion of the N-terminal transmembrane domain (Fig. 7G–I).

TMEM225 is located at chromosome 8q22. A very interesting finding is that 41 olfactory receptor genes (Olr1301 to Olr1341) are also clustered together at this chromosomal locus. TMEM225 is a four-transmembrane protein. It is surprising that the olfactory receptor protein is also a membrane protein with seven transmembrane helices (Sengupta *et al.*, 1996; Dwyer *et al.*, 1998). Whether TMEM225 and olfactory receptor have a relationship in evolution remains to be investigated. Whether TMEM225 plays a role as a receptor similar to the olfactory receptor in the nuclear membrane remains to be identified in our further studies.

The testis is the location of spermatogenesis and sexual hormone production (Lacy and Petitt, 1970). Spermatogenesis is a complicated physiological process including cell

division, differentiation, and interactions between cells in the seminiferous tubule (Wu *et al.*, 2001; Mazzocca *et al.*, 2002; Ohta *et al.*, 2003; Hyenne *et al.*, 2007; Svingen *et al.*, 2007). Some intra- and intercellular events are unique to the testis. These unique functions are likely resulted from the expression of specific genes in the testis. Because spermatogenesis is age dependent, these genes are expressed differentially at various stages of development. Our study results indicated that the transmembrane protein TMEM225 is specifically expressed in the testis, which was supported by the result of bioinformatic analysis showing that all the ESTs of *TMEM225* were transcribed from the testis, and the expression is also age dependent, specifically after adulthood (Figs. 4 and 5). Further, the results of *in situ* hybridization (Fig. 6A, B) showed that *TMEM225* is mainly expressed in spermatocyte cells and round spermatids of seminiferous tubules in adult rat testis. There are two phases in which the testis grow substantially, namely, in embryonic and pubertal age. TMEM225 expression is after puberty stage, at which the testes have already been matured, and the expression phase does not match the testis development stage, which suggests that TMEM225 is not related to the development of testis. The first spermatogenesis wave of male rat was at 44 days after birth. But *TMEM225* mRNA is only detected after the rat is over 1 year old. This suggests that it may not be required for spermatogenesis. The data presented here suggest that TMEM225 may play an important role in sperm degeneration but not in spermatogenesis.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (no. 30700826) and the Medicine Development Foundation of Soochow University (no. EE134709).

Disclosure Statement

No competing financial interests exist.

References

- Aruga, J., and Mikoshiba, K. (2003). Identification and characterization of Slitrk, a novel neuronal transmembrane protein family controlling neurite outgrowth. *Mol Cell Neurosci* **24**, 117–129.
- Best, D., Sahlender, D.A., Walther, N., Peden, A.A., and Adams, I.R. (2008). Sdmg1 is a conserved transmembrane protein associated with germ cell sex determination and germline-soma interactions in mice. *Development* **135**, 1415–1425.
- Cheng, L.J., Li, J.M., Chen, J., Ge, Y.H., Yu, Z.R., Han, D.S., Zhou, Z.M., and Sha, J.H. (2003). NYD-SP16, a novel gene associated with spermatogenesis of human testis. *Biol Reprod* **68**, 190–198.
- Christophe-Hobertus, C., Szpirer, C., Guyon, R., and Christophe, D. (2001). Identification of the gene encoding brain cell membrane protein 1 (BCMP1), a putative four-transmembrane protein distantly related to the peripheral myelin protein 22/epithelial membrane proteins and the claudins. *BMC Genomics* **2**, 3.
- Dwyer, N.D., Troemel, E.R., Sengupta, P., and Bargmann, C.I. (1998). Odorant receptor localization to olfactory cilia is mediated by ODR-4, a novel membrane-associated protein. *Cell* **93**, 455–466.

- Eberhart, C.G., and Wasserman, S.A. (1995). The pelota locus encodes a protein required for meiotic cell division: an analysis of G2/M arrest in *Drosophila* spermatogenesis. *Development* **121**, 3477–3486.
- Endo, K., Akiyama, T., Kobayashi, S., and Okada, M. (1996). Degenerative spermatocyte, a novel gene encoding a transmembrane protein required for the initiation of meiosis in *Drosophila* spermatogenesis. *Mol Gen Genet* **253**, 157–165.
- Fink, C., Weigel, R., Fink, L., Wilhelm, J., Kliesch, S., Zeiler, M., Bergmann, M., and Brehm, R. (2009). Claudin-11 is overexpressed and dislocated from the blood-testis barrier in Sertoli cells associated with testicular intraepithelial neoplasia in men. *Histochem Cell Biol* **131**, 755–764.
- Haugstetter, J., Blicher, T., and Ellgaard, L. (2005). Identification and characterization of a novel thioredoxin-related transmembrane protein of the endoplasmic reticulum. *J Biol Chem* **280**, 8371–8380.
- Huang, C., Zhou, J., Wu, S., Shan, Y., Teng, S., and Yu, L. (2004). Cloning and tissue distribution of the human B3GALT7 gene, a member of the beta1,3-glycosyltransferase family. *Glycoconj J* **21**, 267–273.
- Hulett, M.D., Pagler, E., Hornby, J.R., Hogarth, P.M., Eyre, H.J., Baker, E., Crawford, J., Sutherland, G.R., Ohms, S.J., and Parish, C.R. (2001). Isolation, tissue distribution, and chromosomal localization of a novel testis-specific human four-transmembrane gene related to CD20 and FcepsilonRI-beta. *Biochem Biophys Res Commun* **280**, 374–349.
- Hyenne, V., Harf, J.C., Latz, M., Maro, B., Wolfrum, U., and Simmler, M.C. (2007). Vezatin, a ubiquitous protein of adherens cell-cell junctions, is exclusively expressed in germ cells in mouse testis. *Reproduction* **133**, 563–574.
- Lacy, D., and Pettitt, A.J. (1970). Sites of hormone production in the mammalian testis, and their significance in the control of male fertility. *Br Med Bull* **26**, 87–91.
- Mazzocca, A., Carloni, V., Sciammetta, S., Cordella, C., Pantaleo, P., Caldini, A., Gentilini, P., and Pinzani, M. (2002). Expression of transmembrane 4 superfamily (TM4SF) proteins and their role in hepatic stellate cell motility and wound healing migration. *J Hepatol* **37**, 322–330.
- Morita, K., Furuse, M., Fujimoto, K., and Tsukita, S. (1999). Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. *Proc Natl Acad Sci USA* **96**, 511–516.
- Namiki, M., Kitamura, M., Buczko, E., and Dufau, M.L. (1988). Rat testis P-450(17)alpha cDNA: the deduced amino acid sequence, expression and secondary structural configuration. *Biochem Biophys Res Commun* **157**, 705–712.
- Ohta, H., Tohda, A., and Nishimune, Y. (2003). Proliferation and differentiation of spermatogonial stem cells in the w/wv mutant mouse testis. *Biol Reprod* **69**, 1815–1821.
- Schweneker, M., Bachmann, A.S., and Moelling, K. (2005). JM4 is a four-transmembrane protein binding to the CCR5 receptor. *FEBS Lett* **579**, 1751–1758.
- Sengupta, P., Chou, J.H., and Bargmann, C.I. (1996). odr-10 encodes a seven transmembrane domain olfactory receptor required for responses to the odorant diacetyl. *Cell* **84**, 899–909.
- Svingen, T., Beverdam, A., Bernard, P., McClive, P., Harley, V.R., Sinclair, A.H., and Koopman, P. (2007). Sex-specific expression of a novel gene Tmem184a during mouse testis differentiation. *Reproduction* **133**, 983–989.
- Sze, K.L., Lee, W.M., and Lui, W.Y. (2008). Expression of CLMP, a novel tight junction protein, is mediated via the interaction of GATA with the Kruppel family proteins, KLF4 and Sp1, in mouse TM4 Sertoli cells. *J Cell Physiol* **214**, 334–344.
- Takenaka, M., Yagi, M., Amakasu, K., Suzuki, K., and Suzuki, H. (2008). Retarded differentiation of Leydig cells and increased apoptosis of germ cells in the initial round of spermatogenesis of rats with lethal dwarf and epilepsy (lde/lde) phenotypes. *J Androl* **29**, 669–678.
- Wu, Y., Dowbenko, D., Pisabarro, M.T., Dillard-Telm, L., Koeppe, H., and Lasky, L.A. (2001). PTEN 2, a Golgi-associated testis-specific homologue of the PTEN tumor suppressor lipid phosphatase. *J Biol Chem* **276**, 21745–21753.
- Xing, X.W., Li, L.Y., Liu, G., Fu, J.J., Tan, X.J., and Lu, G.X. (2004). Identification of a novel gene SRG4 expressed at specific stages of mouse spermatogenesis. *Acta Biochim Biophys Sin* **36**, 351–359.

Address correspondence to:
Minghua Wang, Ph.D.

Department of Biochemistry and Molecular Biology
Medical School
Soochow University
Ren-Ai Road 199, Dushu Lake Campus
Suzhou Industrial Park
Suzhou 215123
China

E-mail: mhwang@suda.edu.cn

Received for publication March 16, 2010; received in revised form August 9, 2010; accepted September 1, 2010.