Human t-Complex Protein 11 (TCP11), a Testis-Specific Gene Product, Is a Potential Determinant of the Sperm Morphology

Yanyan Liu, Min Jiang, Chao Li, Ping Yang, Huaqin Sun, Dachang Tao, Sizhong Zhang and Yongxin Ma

Department of Medical genetics & Division of Morbid Genomics, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, P.R. China

Fertilization promoting peptid (FPP) is essential for capacitation and acrosome reaction. The mouse t-complex protein 11 (Tcp11) gene, which encodes the receptor of FPP, plays an important role in fertilization. We had identified three alternative splicing products of its human homologous gene, TCP11, nominated as TCP11a, TCP11b and TCP11c. Their testis-specific expression had been noted, suggesting that TCP11 may play an important role in spermatogenesis and sperm function. In order to explore the function of TCP11, we investigated its expression, subcellular location and binding protein in the sperm. RT-PCR assay shows that all isoforms of TCP11 are present in both human testis and sperm. However, we could only detect the expression of 56-kDa protein, representing TCP11a and TCP11c, but not TCP11b, by western blot analysis. Furthermore, the expression level of 56-kDa TCP11 protein was lower by about threefold in sperm samples containing over 15% of coiled sperms than the level in sperm samples with normal morphology. The coiled sperm, which shows a coiling or bending back of the tail on itself, is associated with infertility. In addition, several TCP11a-binding proteins were isolated using full-length TCP11a as bait. Among them, we focused on outer dense fiber 1 (ODF1), a component of sperm tail outer dense fibers, because outer dense fibers contribute to the distinct morphology and the function of sperm tail. Co-immunoprecipitation assays of sperm cell extracts confirmed that TCP11 protein interacted with ODF1. These results suggest that TCP11 may be responsible for the sperm tail morphology and motility.

Keywords: coiled sperm; leucine zipper; outer dense fiber 1; TCP11; testis


Spermatogenesis is a complex cell differentiation process, during which many drastic changes take place, including formation of the acrosome and sperm tail as well as condensation of the nucleus. Each of these processes requires the highly regulated expression of a number of genes (Willison et al. 1987; Hecht 1995; Sassone-Corsi 1997). In human, the major histocompatibility complex (MHC) provides many candidate genes that could be involved in spermatogenesis, development, embryogenesis and neoplasia (van der Hoorn et al. 1990; Tripodis et al. 1998, 2000). The human MHC is mapped to the short arm of chromosome 6 (Willison et al. 1987; Bibbins et al. 1989; Tripodis et al. 1998). In mouse, this region is the distal inversion of the t-complex of chromosome 17 (Silver 1985). It is only expressed during male germ cell development and found in mouse sperm, particularly in acrosome and flagellum (Fraser et al. 1997). The mouse Tcp11 gene encodes the receptor for fertilization-promoting peptide (FPP) and plays a role in stimulation of capacitation and inhibition of the spontaneous acrosome reaction (Fraser et al. 1997). Human TCP11 is the homologous gene of mouse Tcp11 and is mapped to human chromosome band 6p21 (Ma et al. 2002). We have identified three alternative splicing products of human TCP11, named TCP11a, TCP11b and TCP11c, by homology analysis and subtractive hybridization between the cDNA pools of adult and fetal testes (Ma et al. 2002; Ma et al. 2003a, 2003b). The predicted proteins of TCP11a, TCP11b and TCP11c consist of 441 amino acids, 503 amino acids, and 440 amino acids. These three isoforms differ in their 5′-terminal regions but share the same C-terminal region of 426 amino acids, encoded by 7 exons. In our previous study, we demonstrated that mRNAs of the three human TCP11
isoforms were only expressed in fertile adult testes, but not in other human tissue (Ma et al. 2002; Ma et al. 2003a, 2003b). Here, we report the expression and localization of TCP11 protein in sperm and testis, and its interaction with sperm tail protein ODF1.

Methods

Reagents

Reagents used in this work are TRIZOL Reagent and Lipfectamine2000 (Invitrogen, CA, USA), Random primer SuperScript III Reverse Transcript (TAKARA Biotechnology, Dalian, China), cDNA of normal tissues and adult human testis cDNA expression library (Clontech, CA, USA), mouse polyclonal anti-human TCP11 antibody and HA-tag antibody (Abcam, Cambridge, UK), anti-human ODF1 antibody and Myc-tag antibody (Santa Cruz Biotech Biotechnology, CA, USA), Universal protein extraction buffer (Biotek Corporation, Beijing, China), anti-mouse IgG/FITC, HRP-conjugated anti-mouse IgG and normal goat serum (Zhongshan Goldenbridge Biotechnology, Beijing, China); EDTA-free Protease Inhibitor (Cocktail Roche, Basel, Switzerland), Protein A+G beads (Beyotime, Shanghai, China).

Semen and testes sample preparation

The semen samples were obtained from adult men by masturbation in West China Second University Hospital. All subjects gave written informed consent for scientific evaluation and were with approval of the committee of Ethics of Biological and Medical Research, Sichuan University. Sperm parameters were defined according to World Health Organization criteria. Sperm morphology was evaluated by Modified Papanicolaou staining method, and at least 200 sperm cells were counted in each sample to obtain the normal morphology rate and abnormal morphology rate. We chose 12 semen samples from 12 adult men including 6 infertility patients, whose partners had not conceived spontaneously within 1-2 years and they showed high rate (over 15%) of coiled sperm, for our expression analysis. The rest 6 samples did not contain coiled sperm or contained low rate of coiled sperm. To determine the expression of TCP11 in sperm cells, semen samples were washed through PBS three times at 500 g for 5 min before protein extraction. Fresh testicular tissue from normal adult males who died in accidents was obtained from the West China Hospital, Sichuan University with the consent of the individual’s relatives and with the approval of the Committee of Ethics of Biological and Medical Research, Sichuan University.

RT-PCR to detect the mRNA expression of the three isoforms of TCP11

Total RNA was extracted from sperm and testis using TRIZOL Reagent. The cDNA of normal tissues was purchased from Clontech. RT-PCR was used to detect the mRNA expression of TCP11a (AF269223), TCP11b (AY069943.1), and TCP11c (AF536532) and primers were

TCP11aF (5′-CAACAGCTATGAACCTGAGTCTGG-3′), TCP11aR (5′-TGCACGTCTTCCGGAAAAC-3′), TCP11bF (TG4) (5′-CAGGCCTGTGAAGGGCGGACAC-3′), TCP11bR (R2) (5′-TCTGGCCGTGAATGCTGATACG-3′), TCP11cF (TG4) (5′-CAGTCTGGTCATAGGCCGAAACCT-3′) and TCP11cR (TBR1) (5′-TGGACAGAAAGAATCGTTCTACG-3′) (Ma et al. 2002; Ma et al. 2003a, 2003b). The lengths of predicted cDNA products with TCP11aF + TCP11aR, TCP11bF (TG4) + TCP11bR (R2), and TCP11c F (TG4) + TCP11c R (TBR1) are 322 bp, 325 bp, and 291 bp, respectively.

Western blot analysis

Western blot analysis was used to determine TCP11 protein levels in sperm and testis. Protein extraction from testis and sperm was performed using Universal protein extraction buffers (Biotek Corporation, Bejing, China) containing 5 μl/ml of EDTA-free Protease Inhibitor Cocktail. Protein samples were boiled in sodium dodecyl sulfate (SDS)-PAGE sample buffer and resolved by SDS-PAGE followed by transfer to a PVDF membrane. After transfer, the membranes were blocked in 5% non-fat milk in Tris-buffered saline (TBS) supplemented with Tween-20 (0.1%) for 1 h before being incubated with mouse anti-TCP11 antibody (1 μg/ml) diluted in blocking solution at 4°C overnight. Membranes were washed three times in TBST and then probed with HRP-conjugated rabbit anti-mouse IgG (1:10,000) for 1 h at 37°C. After washing, the specific signals were detected by enhanced chemiluminescence (ECL).

Immunofluorescence analysis

Sperm was dried on glass slides and fixed with 4% paraformaldehyde for 20 min, and then the slides were covered with 10% normal goat serum for 30 min to block nonspecific antibody binding. Subsequently, samples were incubated at room temperature in a humidified chamber for 2 hours with the anti-TCP11 antibody (10 μg/ml). The negative controls were incubated with PBS or non-immune mouse serum. The slides were washed and anti-mouse IgG/FITC diluted 1:100 was added for 1 hour. After that, sperm nuclei were stained with DAPI.

Constructs

HA-tag sequence was added upstream of TCP11a (HA-TCP11a), TCP11b (HA-TCP11b), TCP11c (HA-TCP11c) and deletion mutants of TCP11a respectively, and fused sequences were cloned into pcDNA3.1+ for transfection. A Myc-tag was added upstream of the full-length coding region of ODF1 and the deletion mutants of ODF1 respectively, and fused sequences were cloned into pcDNA3.1+ for transfection too.

Yeast two-hybrid screening

The full-length coding sequence of TCP11a (441 amino acids) was cloned into pGBK7 and used as a bait to screen an adult human testis cDNA expression library. The protocol was performed as the product information described.

Cell culture, Transfection and Co-immunoprecipitation

Human embryonic kidney 293 (HEK293) cells were grown in DMEM plus 10% FBS, and DNA transfection into HEK293 cells was performed using Lipfectamine 2000. HA-TCP11a and Myc-ODF1 were co-transfected into HEK293 cells and the untransfected HEK293 cells were used as a negative control. After protein extraction from the transfected and untransfected cells using Universal protein extraction buffers containing 5 μl/ml of EDTA-free Protease Inhibitor Cocktail, the lysates were centrifuged at 12,000 g for 10 min at 4°C to remove insoluble material and then divided into two equal amounts. One aliquot was exposed to HA-tag antibody and the other was exposed to Myc-tag antibody. Both were incubated at 4°C for 1 hour. Then protein A+G beads were added and the samples were
incubated at 4°C overnight. The complexes were separated by SDS-PAGE followed by Western blot analysis using the HA-tag antibody and Myc-tag antibody. The other co-immunoprecipitations were performed as described above. Endogenous TCP11 and ODF1 were co-immunoprecipitated from sperm lysates by their antibodies and the rest process was performed as described above.

Statistical analysis
For statistical analysis, t test was used. A probability of \( p < 0.05 \) was considered to be statistically significant.

Results
mRNAs of three TCP11 isoforms are expressed in the human sperm

We initially confirmed by RT-PCR analysis that mRNAs of the three human TCP11 isoforms are exclusively expressed in fertile adult testes, but not detectable in other human tissues (Fig. 1), as described previously (Ma et al. 2002; Ma et al. 2003a, 2003b). The expression of \( \beta \)-actin mRNA was used as internal control to confirm the integrity of cDNA synthesis. We next examined whether mRNAs of the three TCP11 isoforms are expressed in sperms by RT-PCR. The result shows that mRNAs of TCP11a, TCP11b and TCP11c are expressed in sperms (Fig. 2A).

Expression of TCP11 proteins in human testis and sperms

The presence of TCP11 isoform mRNAs in testis and sperm does not necessarily imply the expression of TCP11 isoform proteins. Therefore, we analyzed the expression of TCP11 proteins in sperm and testis using Western blot. Interestingly, we could only detect expression of 56-kDa protein, representing TCP11a and TCP11c, but not TCP11b of 63 kDa in human sperm and testis lysates (Fig. 2B). We then tested the specificity of the TCP11 antibody using the lysates of HEK293 cells, which were transfected with each construct of HA-TCP11a, HA-TCP11b and HA-TCP11c. The TCP11 antibody could recognize the HA-TCP11a, HA-TCP11b and HA-TCP11c proteins in transfected cells (Fig. 2B), which was confirmed with the HA antibody (data not shown).

Localization of TCP11 in a human mature sperm

To investigate the localization of TCP11 protein in a mature human sperm, immunofluorescence assay was performed using anti-TCP11 antibody. As shown in Fig. 2C, the signal was detected in head and tail of the mature sperm.

The expression level of TCP11 proteins is low in samples containing high rate of coiled sperm

Western blot analysis was used to evaluate expression levels of TCP11 proteins in various sperm samples. The result showed that TCP11 protein was detected in all sperm cell lysates. Moreover, the level of TCP11 protein in samples, which contained high rate of coiled sperm (\( n = 6 \)), was 70% lower than other ones (\( n = 6 \)) (Fig. 3A and B, \( p < 0.05 \)). The samples containing high rate of coiled sperm were obtained from infertility patients whose partners had not conceived spontaneously within 1-2 years. The rest of samples, which did not contain coiled sperm or contained low rate of coiled sperm, was defined as “other ones”. There were no significant differences in expression of TCP11 protein among the “other ones”. The coiled sperm shows a coiling or bending back of the tail on itself along the length. And high rate of sperm coiling is associated with infertility (Yeung et al. 2009).

ODF1 is a TCP11-binding partner, and leucine zipper is essential for their interaction

To find clues for the molecular mechanism of TCP11, full length of TCP11a protein, the first one discovered in testis by us, was used as a bait in a yeast two-hybrid screening, and several TCP11a-interaction proteins were isolated. One of the identified TCP11a binding proteins was human ODF1 which is a major component of outer dense fibers (ODFs). ODF1 performs an important function for sperm

Fig. 1. RT-PCR analysis of the three types of mRNAs for human TCP11 isoforms in somatic tissues. The expression of mRNAs for three TCP11 isoforms is detected in the testis, but not detectable in prostate, brain, ovary, liver, kidney, heart and pancreas. Quality of cDNAs was confirmed using \( \beta \)-actin specific primers. NC indicates a water control.
tail, so we focus on ODF1 in this study. To explore the in vivo binding of TCP11 protein and ODF1 protein, we performed co-immunoprecipitation assays of sperm lysates. The results indicate that the 56-kDa TCP11 interacts with ODF1 in the human sperm (Fig. 4A).

The molecular mass of human TCP11a protein or TCP11c protein is 56 kDa. Thus, the endogenous isoform proteins were indistinguishable by the co-immunoprecipitation-western blot analysis. Accordingly, we used the fusion protein of TCP11a/c with HA Tag to confirm their physical interaction with ODF1. Reciprocal immunoprecipitation of overexpressed HA-TCP11a and Myc-ODF1 and that of HA-TCP11c and Myc-ODF1 were carried out in HEK293 cells. The results indicated that both HA-TCP11a and HA-TCP11c could interact with Myc-ODF1 (Fig. 4B), further supporting that ODF1 is a binding partner of human TCP11.

Subsequently, we found that TCP11a strongly bound to the ODF1 fragments containing leucine zipper motif, and the deletion of the leucine zipper motif greatly reduced TCP11a-ODF1 association (Fig. 5A-B). The result suggests that the leucine zipper of ODF1 is required for binding to TCP11a.

A previous study showed that binding partners of the N-terminal leucine zipper motif of ODF1 containing one or two leucine zipper motifs (Shao et al. 2001). TCP11a and TCP11c proteins are of 441 and 440 amino acids, and they have the same C-terminal 426 amino acids. The leucine zipper motif is located in the C-terminal 426 amino acids. To detect whether the leucine zipper of TCP11 proteins is involved in association with ODF1 protein, we tested the interaction of ODF1 with each of five TCP11a deletion mutants in HEK293 cells. The results showed that the 171–270 amino acids of TCP11a protein containing the leucine zipper motif interacted efficiently with ODF1, whereas the TCP11a fragments lacking the leucine zipper motif failed to bind to ODF1 (Fig. 5C-D). These results indicate that the leucine zipper motif of human TCP11 protein is essential for association with ODF1.

Discussion

Although previous studies have demonstrated that mRNAs of three TCP11 isoforms are expressed in fertile adult testes, but not in other human tissues. It is still...
Fig. 3. Low expression levels of TCP11 proteins in samples containing high rate of coiled sperm. A. Western blot analysis of TCP11 in samples containing high rate of coiled sperms (lanes 1 and 2), and other sperm samples (lanes 3 and 4). B. Statistical data for the two group samples analyzed by Image J software. Columns 1 and 2 show the coiled sperm sample and the other sperm sample, respectively. The samples containing high rate of coiled sperm were obtained from infertility patients. Other sperm samples contain no coiled sperm or low rate of coiled sperm.

Fig. 4. Interaction between TCP11 and ODF1 in human sperm cells. A. Endogenous TCP11-ODF1 interaction determined by immunoprecipitation assay using sperm extracts. The result showed that the 56-kDa TCP11 protein bound to ODF1 protein. B. TCP11a-ODF1 and TCP11c-ODF1 interaction determined by immunoprecipitation assay using transfected HEK 293 cells. The results indicate that both TCP11a and TCP11c interact with ODF1.
unclear whether TCP11 isoforms are expressed in sperm. In this study, we show that mRNAs of TCP11a, TCP11b, and TCP11c are expressed in the human sperm. Interestingly, the protein of TCP11b is absent in human sperm and testis lysates. Moreover, the localization of 56-kDa TCP11 is in the head and flagellum of the mature sperm.

Expression level of TCP11 is 70% lower in samples containing high rate of coiled sperm than that in healthy sperm samples. It has been revealed in a long-term follow-up study that infertile patients who are eventually proven fertile show lower rate of sperm coiling (9%) than persistently infertile patients (11%) (Jouannet et al. 1988), and both percentages are higher than in fertile men (7%) (Auger et al. 2001). This suggests that sperm coiling is associated with infertility and that sperm coiling could compromise the fertilizing capacity of sperm (Yeung et al. 2009). While the expression of TCP11 is lower in samples with high percentage of sperm coiling, the role of reduced TCP11 expression as a consequence or reason for the coiled sperm remains unclear.

To investigate the function of TCP11, we performed yeast two-hybrid screening to identify TCP11 binding partners. One of the TCP11 binding proteins was ODF1, a major component of the outer dense fibers (ODFs). ODF1 is the first component to be identified of ODFs, which contribute to the distinctive morphological features of the sperm’s motile apparatus and protect the sperm tail against shear forces during transport and ejaculation (Baltz et al. 1990). To explore the possible protein interactions related to sperm tail development and function, we carried out co-immunoprecipitation and proved the association between ODF1 and 56-kDa TCP11 protein. Furthermore, our results reveal that both TCP11a and TCP11c interact with ODF1. And leucine zipper motif is essential for their interaction. The previous works proposed that ODF1 plays a role in

**Fig. 5. Interaction between HA-TCP11 and different ODF1 deletion mutants.**

**A.** Interaction between HA-TCP11 and different ODF1 deletion mutants (M1-M3) with Myc tag. **B.** Schematic representation of ODF1 deletion constructs. The results indicated that TCP11a strongly bound to the ODF1 fragments containing leucine zipper motif (M3) and the deletion of the leucine zipper motif (M1, M2) greatly reduced TCP11a-ODF1 association. **C.** Interaction between Myc-ODF1 and different TCP11 deletion mutants with HA tag (C1-C5). **D.** Schematic representation of TCP11a deletion constructs. These results indicate that the 171–270 amino acids of TCP11a protein containing the leucine zipper motif (C3) interacted efficiently with ODF1, whereas the TCP11a fragments lacking the leucine zipper motif failed to bind to ODF1.
sperm tail morphogenesis by providing the basis of a complex protein network (Willison et al. 1987; Zarsky et al. 2003). Interaction of TCP11 with ODF1 suggests that TCP11 may be a member of this protein network and TCP11 might contribute to the distinct morphology and function of the sperm tail.

In conclusion, our study is the first report on the expression pattern of three isoforms of human TCP11 in sperm. Our data provide new insights into our understanding of the function of TCP11 and the TCP11-ODF1 complex in sperm morphology and sperm motility.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31070676, 30770812 and 90919006) and National High-Tech Research and Development Program of China (2008AA02Z102).

Conflict of Interest

The authors report no conflict of interest.

References


