Testis-Specific Gene *C7orf61* Is Involved in Mouse Sperm–Egg Fusion

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Purpose: Chromosome 7 open reading frame 61 (C7orf 61) was a testis-specific gene, and may be involved in the process of spermatogenesis. This study aimed to investigate the expression of C7orf61 in the testis and determine its role in spermatogenesis.

Materials and Methods: Reverse transcription–quantitative polymerase chain reaction, Western blot and immunofluorescence were performed to evaluate the expression characteristics of C7orf61 in mice and humans. In vitro fertilization assay was used to determine the role of the C7ORF61 protein in sperm-egg fusion.

Results: The results demonstrated that C7orf61 was a testis-specific gene; the C7ofr61 mRNA expression level sharply increased in the fourth postnatal week and gradually increased until the adult stage. The C7ORF61 protein was located throughout the subacrosomal area and close to the nucleus in both mouse and human sperm. The incubation with the C7ORF61 antibody significantly decreased the fertilization rate of mouse eggs.

Conclusion: The present findings suggested that the C7ORF61 protein might be involved in sperm–egg fusion, and could serve as a useful target for contraceptives. However, further research is still needed to know the detailed molecularmechanismofitsrole.

Keywords: *C7orf 61*; sperm–egg fusion; testis; spermatogenesis; sperm

INTRODUCTION

The three-child policy in China has increased the focus on male reproductive health. The quality of male semen has declined, and the number of infertile male patients continues to increase every year with the improvement in social industrialization, aggravation of environmental pollution, bad living habits, and other factors(1,2). Infertility has become the third largest disease after cancer and cardiovascular disease, and male infertility accounts for 40% to 50% of the cases.

Spermatogenesis is a complex developmental process regulated by a series of genes with testis-specific expression in an orderly spatiotemporal manner. The proliferation and differentiation of spermatogonia involve mitosis, meiosis, transformation of round sperm cells into elongated sperm cells, and finally the formation of mature sperm in the epididymis(3). The transformation of round sperm cells into elongated sperm cells requires fine and accurate nuclear remodeling and the formation of specific cell structures, including apical cytoplasmic specialization and acrosome formation. Any abnormality leads to abnormal spermatozoa and abnormal sperm–egg fusion(4). Therefore, the in-depth study of the molecular mechanism of sperm–egg fusion is of great scientific significance for clarifying the cause of male infertility and identifying therapeutic targets for sperm–egg fusion disorder.

In the previous study, we analyzed the high-throughput gene expression profile chip data (http://biogps.org) and expressed sequence tags (ESTs) expression profile (http://www.ncbi.nlm.nih.gov/UniGene, Mm. 56514), and found that chromosome 7 open reading frame 61 (C7orf 61) was a testis-specific gene. The mouse gene is located on chromosome 5 and consists of 6 exons. C7orf 61 mRNA (NM_ 001395754.1) is 1778 bp, and encodes a protein composed of 210 amino acids (NP_ 001382683.1). A study reported that C7orf61 was a new candidate gene for human round-headed spermatozoa(5). UniProt showed that C7ofr61 may play a role in acrosome formation and nucleus shaping during spermatogenesis. Collectively, C7orf61 has a critical role in the male infertility, but the function and molecular mechanism remain unclear and need further investigation.

MATERIALS AND METHODS

Samples

C57BL/6 mice and SD rats were purchased from Cyagen Biosciences (Suzhou, Jiangsu, China). All the animal experimental protocols were reviewed and approved by the ethics committee of Jingzhou Hospital Affiliated to Yangtze University (No. 202003-001, Jingzhou, Hubei). Human samples were from biological sample bank of Peking University Shenzhen Hospital. Testis tissue

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Table 1. Primers for genes used in the study.

Gene	Primer (5'-3')	Product Size
Mouse C7orf61	F: TCCTCAGTACCCGCAAGTCC R: CCTGGCTTGGCTAGGTGTTT	206 bp
Mouse β-Actin	F: GCAGATGTGGATCAGCAAGC R: AGGGTGTAAAACGCAGCTCAG	102 bp
Human C7ORF61	F: CCAACAGGGCCAATCCCTAC R: CAAAGGTGACTGCGTCCTCA	160 bp
Human β-ACTIN	F: CCTTGCACATGCCGGAG R: GCACAGAGCCTCGCCTT	112 bp

array was bought from Bioaitech. com (Xi'an, China). The wild-type postnatal testes aged 1to 8 weeks were collected from at least three C57BL/6 mice. Other organ samples of adult mice used in the study were from 8-9 weeks old C57BL/6 mice (n = 6). The experimental mice and rats were kept at stable SPF condition for at least 5 days with water and chow ad libitum. Light/dark cycle was set at 16-h/8-h, and room temperature att 22°C to 25°C and relative humidity at 50% to 60%. Euthanasia was performed by cervical dislocation after intraperitoneal injection of pentobarbital sodium.

Reverse transcription–quantitative polymerase chain reaction

TRIzol (Thermo Fisher Scientific, Inc.) was used to extract total RNA from mouse testes, and the PrimeScript RT Master Mix kit (Takara Bio, Inc.) was applied to perform cDNA synthesis following the manufacturer's protocol. The SYBR Premix EX TaqTMII PCR kit (Takara Bio, Inc.) was used to perform reverse transcription–quantitative polymerase chain reaction (RT-qPCR), with gene-specific primers. The detailed information for the primers is listed in Table 1. The annealing temperature was 60°C, with 40 cycles. The data were calculated using the $2^{-\Delta\Delta}$Cq method[6].

In vitro fertilization

Following a previous study[7], the cauda epididymitis of wild type mice was removed and sheared in PBS to release sperm. Mouse sperm were collected by centrifuging. The capacitation of sperm was induced in a 200 μL drop of human tubal fluid (HTF; Millipore) supplemented with 10% BSA, which was covered by mineral oil (Sigma). After incubation in vitro for 1.5 h, 20 μg/mL anti-C7ORF61 antibody was added into the drop and incubated at 37°C for 1 h in cell culture incubator. Normal rabbit IgG (sc-2027, Santa Cruz Biotechnology) at the same concentration were used as controls. Then, 8- to 9-week-old C57BL/6J female mice were subjected to superovulation, which was induced by administering intraperitoneal (i.p.) injections of 7.5 units of pregnant mare serum gonadotropin (PMSG; Sigma), 48 h later i.p. injection of 7.5 units of human chorionic gonadotropin (hCG; Sigma). 13 h later, the cumulus-intact eggs were collected from the swollen ampulla. The eggs were washed and inseminated with 1 × 106 sperm/mL for 6 h at 37°C. The eggs were transferred to the M16 medium (Sigma) drops containing 10% BSA, and cultivated undisturbed for 1 day. Progression to the two-cell stage was defined as developing embryos.

Immunofluorescence

The protocol was followed a previous study with minor revision[8]. Mouse and rat testes were removed and washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde at 4°C for 24-48 h. Then the tissue was embedded in paraffin, and cut into 3 μm sections. The slides were subjected to dewaxing and rehydrating. For antigen retrieval, the sections were immersed in 10 mM sodium citrate (pH 6.0) and microwaved for 30 min (high for 5 min, and low for 25 min). After cooling down to room temperature, the slides were treated with 0.05% Triton X-100 for 15 min, and then blocked in 10% BSA for 30 min. An anti-C7ORF61 antibody (1: 200; cat. no. NBP1-56948; NOVUS) was added to the sections and maintained at 4°C. After three washes with PBS, the slides were incubated with an anti-rabbit Alexa Fluor 488 antibody (1: 2,000; cat. no. A-27034; Thermo Fisher Scientific, Inc.) at 37°C for 1h. Hoechst 33342 (1: 2,000; Invitrogen; Thermo Fisher Scientific, Inc.) were added to the slides for counterstaining for 5 min. With two additional PBS washes, the slides were mounted in SlowFade (Invitrogen; Thermo Fisher Scientific, Inc.), covered with nail polish. Finally, the slieds were observed using a

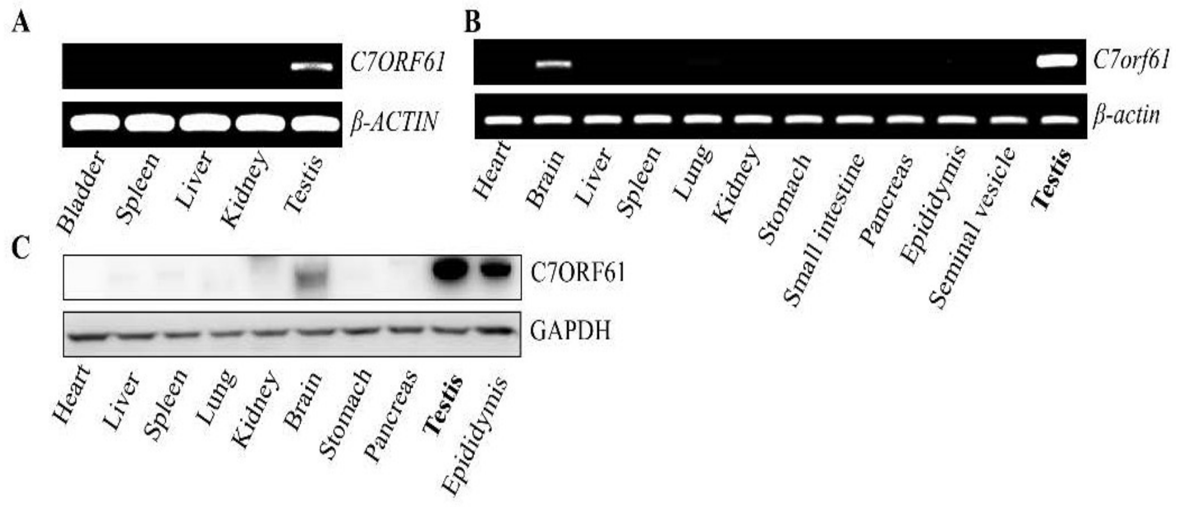

Figure 1. Expression of C7ofr61 in mouse and human tissues. The presence of C7ofr61 mRNA was evaluated in various tissues of adult human and mice using RT-PCR. C7ofr61 mRNA was detected in the human **(A)** and mouse testes **(B)**. β-Actin was amplified as an internal control. The expression of C7ORF61 in mouse tissue was determined by Western blot **(C)**. Protein expression was normalized by GAPDH.

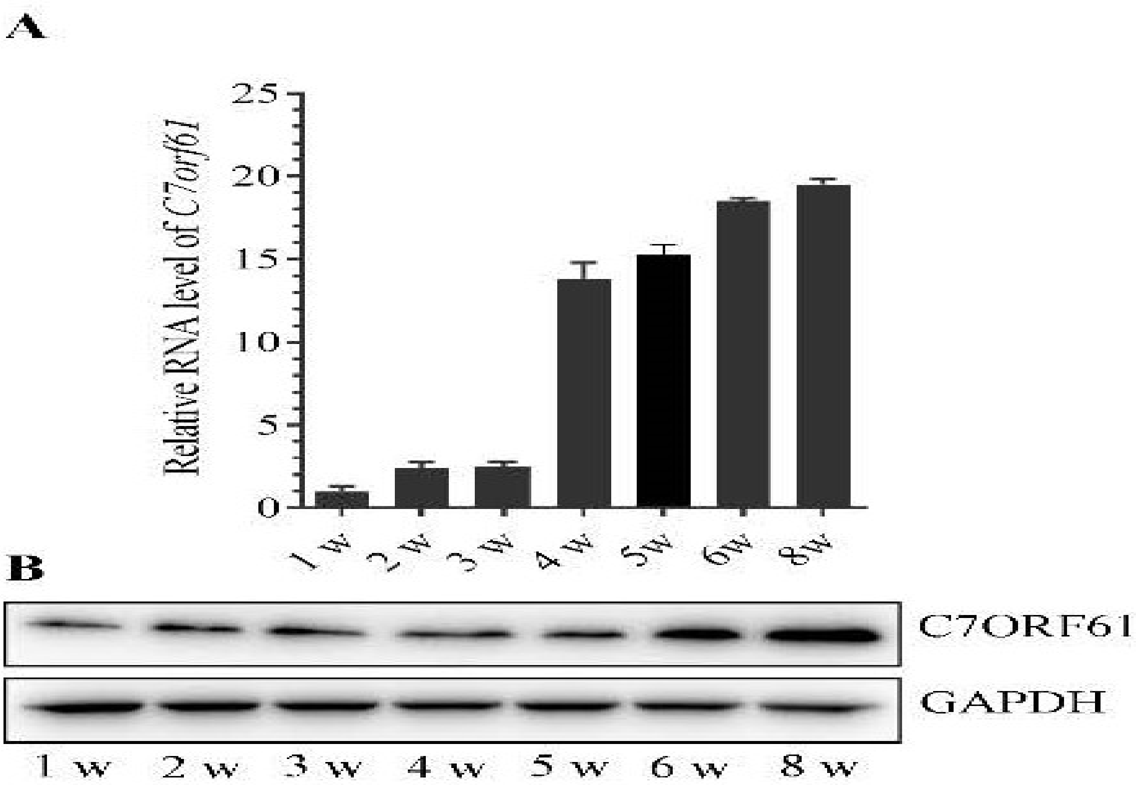

Figure 2. Temporal expression of C7ofr61 during the postnatal development of mouse testis. The testicular C7ofr61 expression profile was tested at the indicated time points after birth using RT-qPCR **(A)** and Western blot **(B)**. C7ofr61 mRNA expression acutely increased after 4 weeks, and the protein levels increased by 6 weeks. Data are presented as mean ± SD (n = 5).

fluorescent microscope (magnification, 200 × ; Zeiss GmbH).

Immunohistochemistry

Immunohistochemistry was conducted following the instruction provided by the DAB kit (PV-6000-D, ZSGB-Bio, China). The tissue array was subjected to dewaxing and antigen retrieval as described previously. The slides were incubated with endogenous peroxidase blocking solution for 15 min at room temperature. After three washes with PBS, anti-C7ORF61 antibody (1: 200; cat. no. NBP1-56948; NOVUS) was added and maintained at 37 C for 1 h. Then the slides were incubated with horseradish linked goat anti-rabbit IgG for 15 min. DAB staining reagent was added to the slides and incubated for 5 min following repeated washes with PBS. The slides were counterstained with hematoxylin, and then dehydrated, transparent, and mounted by neutral balsam mounting medium.

Western blot

Protein samples of different tissues including heart, liver, spleen, lung, kidney, brain, stomach, pancreas, testis, and epididymis from mice, were extracted using RIPA lysis buffer (Beyotime), then 20 µg protein samples were loaded and run on 4-20% SDS-PAGE. After transferring onto a polyvinylidene fluoride (PVDF) membrane, we used 10% (w/v) non-fat milk in TBS buffer with 0.05% Tween-20 (Sigma-Aldrich) to block the membranes for 1 h at room temperature. After two washed with TBS, the membranes were incubated with primary antibodies targeting C7ORF61(Novus; cat. no.NBP1-56948; 1: 1,000 dilution), GAPDH (Cell Signaling Technology, Inc.; cat. no. 5174; 1: 1,000 dilution) overnight at 4˚C. The following day, with another three washed with TBS, the membranes were incubated with horseradish peroxidase-labeled secondary antibody (anti-rabbit; 1: 1,000; cat nos. 7074, Cell Signaling Technology, Inc.) for 1 h at room temperature. An enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.) was applied to detect the positive bands.

Declarations

All experimental protocols involving animals and human tissues were reviewed and approved by the Ethics Committee of Jingzhou Hospital Affiliated to Yangtze University (No. 202003-001, Jingzhou, China). All the methods performed were in accordance with relevant guidelines and regulations, as well as the principles of the Declaration of Helsinki.

Statistical analysis

All of the experiments were performed at least three times. The data were expressed as mean ± standard deviation (SD) in Figures 2A and 6B. SigmaPlot 16.0 (Systat Software, Inc.) was applied to conduct statistical analysis, and statistical significance was evaluated using one-way analysis of variance after the assenment of the normality and homogeneity of variance, and followed by Fisher's protected least-significant difference post hoc test, unless otherwise specified. A P value < .05 was set as a statistically significant difference.

RESULTS

Expression characteristics of the C7orf61 gene in different tissues of mice and humans

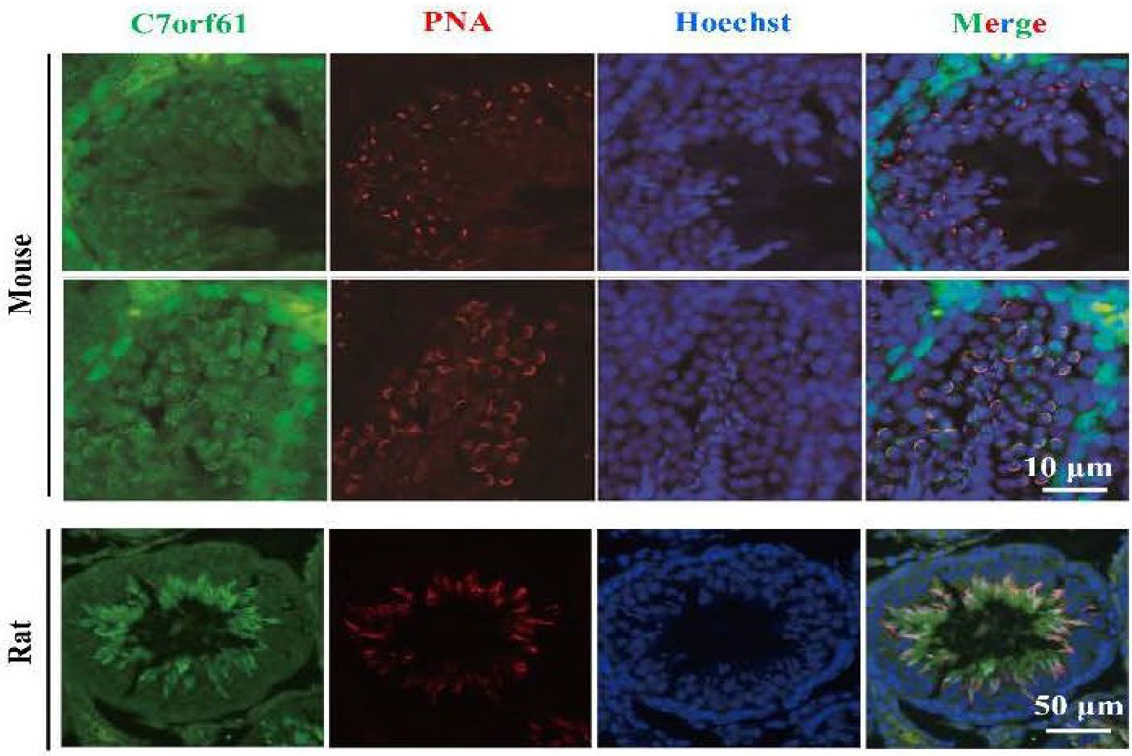

Figure 3. Immunofluorescence staining for the C7ORF61 protein in mouse and rat testis. Testes sections were subjected to immunofluorescence microscopy using an anti-C7ORF61 antibody (green), PNA (red, Alexa FluorTM 594 conjugated, Cat No. L32459, 1: 200 dilution, Invitrogen), and Hoechst staining (blue), as described in the Materials and Methods section.

RNA-seq data of tissue samples from 95 human individuals representing 27 different tissues (https://www.ncbi.nlm.nih.gov/gene/402573), which was designed to determine tissue-specificity of all protein-coding genes, showed C7orf61 was predominantly expressed in the testis. The expression of C7ofr61 mRNA in various tissues of mice and humans was determined by RT-qPCR. As shown in **Figure 1A and 1B**, C7ofr61 was exclusively expressed in the human and mouse testes, which agreed with the EST profile in the Unigene database. This was confirmed by the Western blot data in **Figure 1C**.

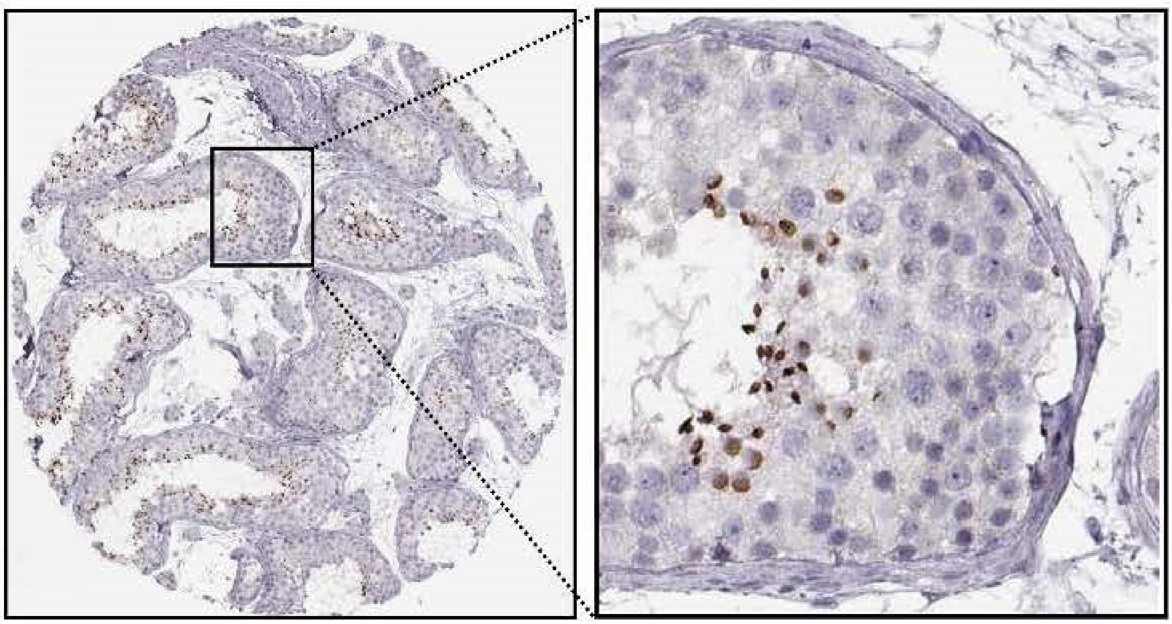

Figure 4. Localization of the C7ORF61 protein in human samples. Immunohistochemistry assay showed C7ORF61 protein was expressed mainly in elongated spermatids and mature sperm.

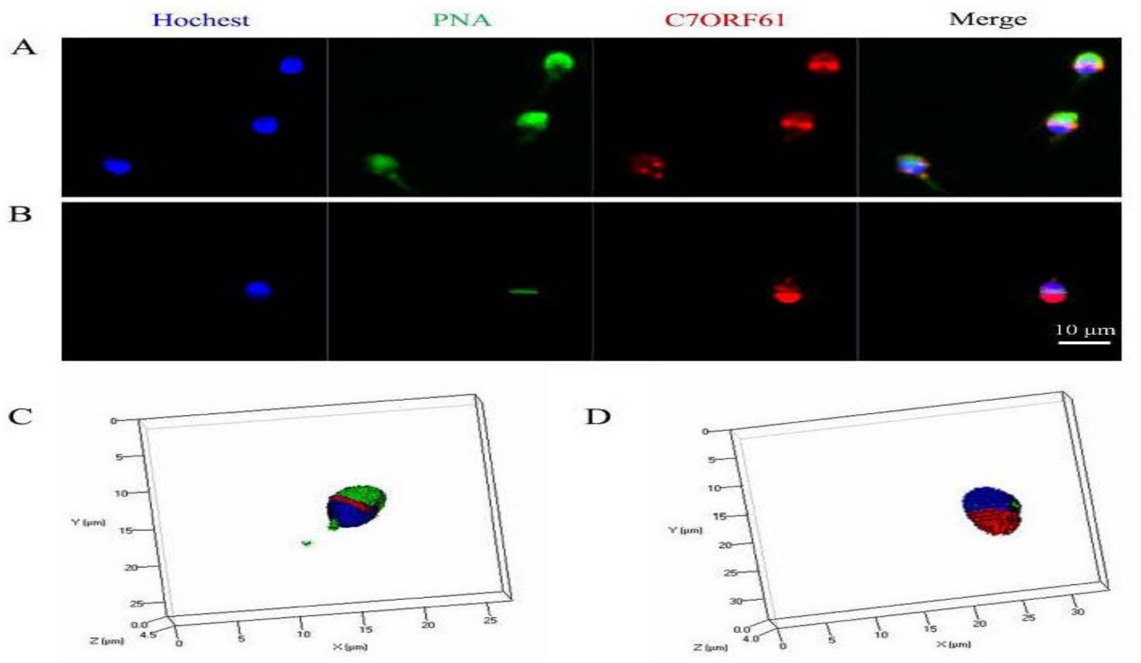

Figure 5. Localization of C7ORF61 protein in human sperm. Immunofluorescence co-localization and three-dimensional imaging technology were used to detect the localization of the C7ORF61 protein in human sperm before the acrosome reaction **(A and C)** and after the acrosome reaction **(B and D)**. Hoechst (blue), PNA (green, Alexa FluorTM 488 conjugated, Cat No. L21409, 1: 200 dilution, Invitrogen), and C7ORF61 (red). Scale bars = 10 μm.

Expression levels of C7orf61 during the development of mouse testis

The RT-qPCR analysis of postnatal mouse testes aged 1-8 weeks showed that the C7ofr61 mRNA expression level sharply increased in the fourth postnatal week, with the first wave of spermatogenesis, just before the initiation of elongation of spermatid, and the expression level was gradually increased until the adult stage (**Figure 2**).

C7ORF61 expression during spermatogenesis

Immunofluorescence analysis was carried out to identify the initial expression and location of C7ORF61 protein in the mouse and rat testis. As shown in Figure 3, the C7ORF61 protein was expressed mainly in elongated spermatids, but not colocalized with peanut agglutinin (PNA), a sperm acrosome-specific marker. Localization of C7ORF61 protein in human testis array Immunohistochemistry assay of human testis confirmed that C7ORF61 protein was expressed mainly in elongated spermatids and mature sperm (**Figure. 4**), suggesting an important role of C7ORF61 protein in the spermatogenesis. Expression profiling by an array of 21 samples (https://www.ncbi.nlm.nih.gov/geoprofiles/38151368) showed that teratozoospermic individuals (8 samples) had lower expression of C7ORF61 protein than normospermic individuals (14 samples).

Localization of C7ORF61 protein in human mature sperm

The expression characteristics of the C7ORF61 protein in human sperm before the acrosome reaction (Figure 5A and 5C) and after the acrosome reaction (Figure 5B and 5D) were detected and compared using immunofluorescence co-localization and three-dimensional imaging technology. The C7ORF61 protein was found to be located below the sperm acrosome-specific marker PNA and above the nuclear-specific marker signal Hoechst. After the acrosome reaction, the sperm head above the equatorial plate was displayed. Thus, the C7ORF61 protein was located in the subacrosomal region of human sperm and close to the nucleus.

Anti-C7ORF61 antibody affected the fertilization rate of mice in vitro

Gene Ontology (GO) analysis showed a potential role of C7orf61 in microtubule binding (**Figure 6**). Taken together with the localization of the C7ORF61 protein, we speculated that it might be related to sperm-egg fusion. After incubating the C7ORF61 antibody or goat anti-rabbit immunoglobulin G (IgG) with mouse mature sperm, fertilization and cleavage were observed. As shown in representative figures in **Figure 7A** and data analysis in Figure 7B, the C7ORF61 antibody significantly decreased the fertilization rate and cleavage rate of mouse eggs. The two-cell cleavage rate after 24 h in the anti-C7ORF61 group was significantl lower than that in the vehicle and control IgG groups.

DISCUSSION

Sperm-egg fusion is a cell-cell membrane fusion event of sperm and egg, and it is essential for the propagation of sexually reproducing organisms[9]. However, the detailed molecular mechanism involving gamete fusion remains largely unknown[10]. Only a few proteins required for membrane interaction are identified and generally recognized, including IZUMO1[11], present in the sperm; tetraspanin CD9[12], present in the egg; and the newly found oolema protein, named Juno[13].

It was reported that the C7orf61 is a known round spermatid marker that belonged to the top differentially expressed genes of Round SPT1[14], which is a subcluster of round spermatids by single cell transcriptome se-

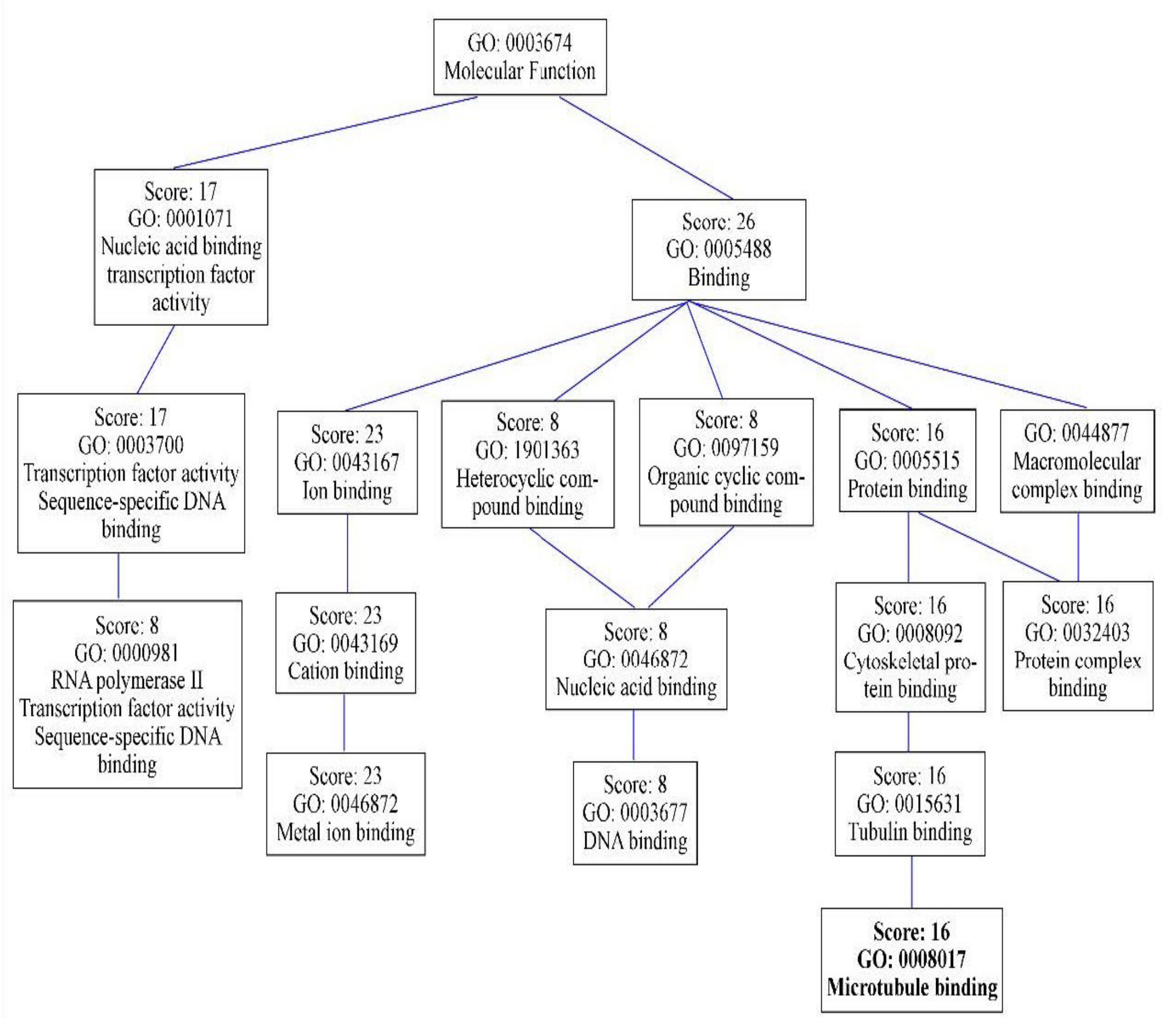

Figure 6. Gene Ontology analysis of C7orf61.

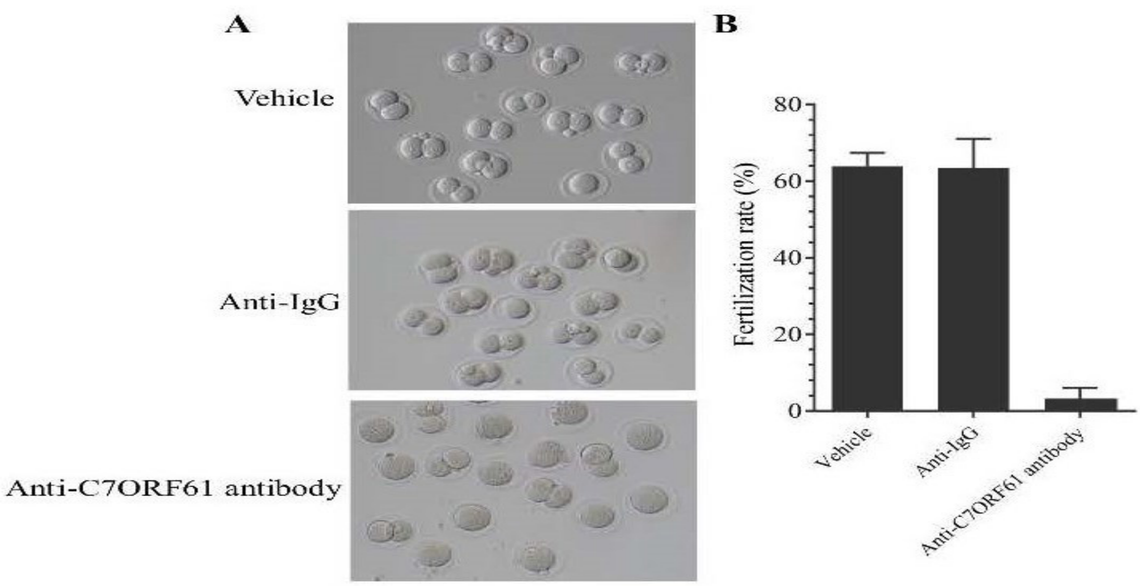

Figure 7. Decreased fertilization rate in anti-C7ORF61 antibody-treated mouse sperm. Cumulus-intact eggs were inseminated with caudal sperm treated with vehicle, IgG, or anti-C7ORF61 antibody. **(A)** Represented figures of the putative fertilized oocytes from three groups. **(B)** Collected data from repeated experiments, data are presented as mean ± SD (n = 3).

quencing. The present study showed that the C7orf61 gene had testis-specific expression in both human and mouse testes.

Another study also suggested a critical role of C7orf61 in the pathogenesis of globozoospermia; mutations in C7orf61 ((c.259del; p.(Glu87Argfs*46)) were reported in patients with globozoospermia(5,15-17). Consistent with these findings, the expression of C7orf61 mRNA sharply increased in the fourth postnatal week until adulthood in this study. The C7ORF61 protein was found to be located throughout the subacrosomal area of mouse mature sperm and close to the nucleus. Western blot also showed C70RF61 protein in the brain, this could be explained that brain and testis shared a similar gene expression profile(18-20). RT-PCR did not show any amplification in epididymis of the gene, whereas protein expression was evident may be due to the protein located in the sperm transferred from testis.

Immunofluorescence co-localization and three-dimensional imaging technology confirmed the localization of the C7ORF61 protein in human sperm. The ultrastructural analysis of spermatozoa in patients with C7orf61 mutation revealed a mixture of round heads with no acrosomes and ovoid or irregular heads with small acrosomes frequently detached from the sperm head(5). In our lab's previous study, SPATA46(7) and FAM170B (21) were identified and proved to be involved in reshaping of the sperm head and sperm-egg fusion. Similar to these proteins, the C7ORF61 protein may play an important role in acrosome formation and nucleus shaping during spermatogenesis and can be considered a novel candidate gene for acrosomal hypoplasia.

In the present study, the sperm were treated with vehicle, IgG, or anti-C7ORF61 antibody to figure out the role of the C7ORF61 protein in male fertility. The cleavage rate 24 h after insemination showed that the C7ORF61 protein could significantly inhibit sperm–egg fusion in mice. This data suggested that the C7ORF61 protein played an important role in sperm-egg fusion. This protein might serve as a useful target for contraceptives. However, further studies are required to verify and clarify the effect of C7ORF61 in male fertility.

CONCLUSIONS

In conclusion, the present study demonstrated that C7orf61 was a gene with testis-specific expression. The C7ORF61 protein was located throughout the subacrosomal area and close to the nucleus, and the blockade of this protein significantly decreased the cleavage rate. Therefore, the C7ORF61 protein may play a potentially important role in spermatogenesis and also in sperm-egg fusion. Further studies are needed to better understand the molecular mechanisms of C7orf61 in sperm-egg fusion.

FUND

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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