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Discovery of a novel miRNA involved in the regulation of male infertility in zebrafish

Wen Fu [a,b,c], Feng Liu [a,c], Yingying Wang [a,c], Ze Li [a,c], Wenpei Deng [c], Wenbin Liu [a,b,c], Jinhui Liu [a,b,c], Liangyue Peng [a,b,c,*], Yamei Xiao [a,b,c,*]

[a] State Key Laboratory of Developmental Biology of Freshwater Fish, Hunan Normal University, Changsha 410081, China
[b] Engineering Research Center of Polyploid Fish Reproduction and Breeding of the State Education Ministry, Changsha 410081, China
[c] College of Life Sciences, Hunan Normal University, Changsha 410081, China

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ABSTRACT

Azoospermia and asthenospermia are common manifestations of male infertility, but it needs further studies to understand the intrinsic regulation mechanism. As a popular model organism, zebrafish is often used to assess reproductive complications. In this study, by analyzing miRNA transcriptome of the mature triploid zebrafish testis afflicted with spermatogenic dysfunctions, leading to the identification of 36 miRNAs that are differentially expressed in comparison with diploid, which are predicted to target 2737 genes. Subsequent functional annotation of these genes pinpointed two miRNAs might association with spermatogenesis. Inhibitory experiments showed that NC_007115.7.7_998413 inhibited conducts a substantial decline in sperm density, and conducted lower embryo fertilization rate than control. And putative target genes qRT-PCR evaluation showed that *spata2* was significant down-regulate upon inhibited NC_007115.7.7_998413. In summary, this research positions newly identified miRNA NC_007115.7.998413 as a regulatory factor in male zebrafish reproductive development, enhancing our comprehension of the molecular regulated pathways involved in spermatogenesis.

1. Introduction

Clinical studies have indicated that about 10–20% of couples in the world are infertile, with male factors implicated in 30–50% of infertility cases [1]. The most common clinical manifestations of the male infertility are idiopathic non-obstructive azoospermia (NOA) and severe oligoasthenospermia, often caused by abnormal spermatogenesis in the testis [2–4]. Fertility has been becoming a crucial research topic in the fields of biology and medicine since the infertility has emerged as one of the top three health concerns in the 21st century [5–7]. Therefore, in view of improving male reproductive health and population quality, it is valuable to extensively and deeply investigate the molecular regulation mechanism of spermatogenesis disorders in individuals with NOA and severe oligoasthenospermia.

Fish belong to the lower vertebrates, which cover almost all the known diverse and complicated reproductive patterns of the vertebrates [8]. Triploid fish have three sets of chromosomes, and their gonads often suffer from impaired development or aneuploid gametes since their chromosomes are difficult to pair properly, consequently, the triploid fish are generally sterile [9,10]. For example, in the triploid *Ictalurus punctatus* [11], *Cyprinus carpio* [12] and *Scophthalmus maximus* [13], their testis are unable to produce mature sperm. The triploid *Gadus morhua* [14] and *Takifugu niphobles* [15] produced only a small number of aneuploid sperm. The male triploid *Pagrus major* generated only euploid gametes, but their sperm density was approximately one-tenth of that observed in the diploid ones. The sperm of triploid *Pagrus major* exhibited reduced activity and were less flexible and robust compared to those of the diploid [16]. Zebrafish is a popular model organism, and owing to its complete available genetic database, it has been widely used in the study of vertebrate gene function and human genetic diseases [17,18]. Approximate 70% of human genes possess at least one identifiable zebrafish ortholog in comparison to the human reference genome, which also enhances the credibility and potential significance of using zebrafish as a research model for human genetic diseases [19–21]. Notably, previous studies have shown that the male adult triploid zebrafish prepared by heat shock displayed significant spermatogenesis disorders, which only yielded small amounts of water-like semen [22]. However, the regulatory mechanism underlying

* Corresponding authors at: State Key Laboratory of Developmental Biology of Freshwater Fish, Hunan Normal University, Changsha 410081, China.
E-mail addresses: ply@hunnu.edu.cn (L. Peng), yameix@hunnu.edu.cn (Y. Xiao).

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spermatogenesis disorders in triploid zebrafish remains unclear.

MicroRNAs (miRNAs) play a pivotal role in the regulation of male spermatogenesis, with their expression varying significantly across different stages of spermatogenesis and types of spermatogenic disorders. In addition, miRNA had high stability, strong tissue expression specificity and high sequence conservation, which made it had great potential in the diagnosis of spermatogenesis disorders [23]. In this research, through miRNA sequencing of the mature testis from both triploid and diploid male zebrafish, we attempted to identify the key candidate miRNAs implicated in the regulation of spermatogenesis. Subsequently, the functional roles of these pivotal miRNAs would be elucidated, elucidating their regulatory mechanisms in the spermatogenic anomalies of triploid zebrafish. Ultimately, our findings could provide meaningful insights for the management of conditions like oligospermia and asthenospermia.

2. Materials and methods

2.1. Experimental materials

The triploid zebrafish used in this study were prepared by heat shock [22]. The diploid (AB strain) and triploid zebrafish were both raised in the State key Laboratory of Freshwater Fish Developmental Biology at Hunan Normal University in China, and the adult zerafish were raised in an independent aquaculture system (ESEN, China). The water temperature was controlled in the interval (27–29 °C), and the photoperiod was fixed at 14 h of light and 10 h of darkness.

2.2. RNA isolation, cDNA library construction, and sequencing

Total RNA was isolated from the testis of adult diploid and triploid zebrafish tissues using the Trizol Reagent (TaKaRa, China) in line with the manufacturer's protocol. RNA integrity was assessed by the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA), to ensure qualified samples for sequencing. A total amount of 2.5 ng RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated by NEBNext^{R} Ultra™ Directional RNA Library Prep Kit for Illumina^{R} (NEB, USA) subject to the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. PAGE gel was used for the electrophoresis fragment screening purposes, rubber cutting recycling as the pieces get small RNA libraries.Then, the PCR products were purified (AMPure XP system) and the library quality was assessed by the Agilent Bioanalyzer 2100 system. The libraries were sequenced using Illumina HiSeq Xten, at BMK Beijing, China. All the raw data of the testis libraries were deposited in the NCBI SRA (Accession Number: PRJNA996758).

2.3. Comparative analysis

Use Bowtie tools soft, The Clean Reads respectively with Silva database, GtRNAdb database, Rfam database and Repbase database sequence alignment, filter ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA) and other ncRNA and repeats. The remaining reads were used to detect the known miRNA and the novel miRNA predicted by comparing with known miRNAs from miRBase. Randfold tools soft was used for the novel miRNA secondary structure prediction.

2.4. Differential expression analysis

Differential expression analysis of two conditions/groups was performed using the edgeR R package. This package can provide statistical routines for determining differential expression from the digital miRNA expression data using a model based on the negative binomial distribution. The resulting *P* values were adjusted using the Benjamini and Hochberg's approach to control the false discovery rate. The miRNA with an adjusted $p < 0.05$ found by the software package edgeR were assigned as differentially expressed.

2.5. Prediction of differentially expressed miRNA target genes

According to the known differentially expressed miRNA and the zebrafish reference genome sequences (ftp://ftp.ncbi.nlm.nih.Gov/genomes/all/GCF/000/002/035/GCF000002035.6_GRCz11), target gene prediction was performed using miRanda [24] and RNAhybrid [25]. The software Cytoscapez was used to make a visual network diagram of interaction between differentially expressed miRNA and target genes.

2.6. Target gene functional annotation

Genes function was annotated by PSI-BLAST against the protein databases of Nr (NCBI non-redundant protein sequences) ; Nt (NCBI non-redundant nucleotide sequences) ; Pfam (Protein family) ; KOG/COG (Clusters of Orthologous Groups of proteins) ; Swiss-Prot (A manually annotated and reviewed protein sequence database) ; KO (KEGG Ortholog database) ; GO (Gene Ontology).

2.7. Quantitative real-time PCR analysis (qRT-PCR)

The total RNAs, extracted from the remaining tissue, were used for qRT-PCR analysis. These RNA samples were first treated by Mir-X miRNA First-Strand Synthesis Kit (Takara, China) to remove residual genomic DNA and reverse transcribe into cDNA. Then, the qRT-PCR analysis was performed using the Prism 7500 Sequence Detection System (Applied Biosystems) with a miScript SYBR Green PCR kit (Selleck, China). Fold changes in expression were calculated using the $2^{-\Delta\Delta Ct}$ method. Each sample, qRT-PCR analysis was done by three biological replicates. Primers for qRT-PCR were shown in table S1 in Supplementary.

2.8. miRNA inhibition

Antagomir (GenePharma, China) is a highly effective blocker designed according to the mature sequence of miRNA (Antagomir NC_007115.7_998413: 5'-GCC AGC CAG CCA GCC CAG GCC A-3′, Antagomir NC_007131.7_4522250: 5'-GCC CAC CCA CCA CAT CCC CA-3′). Antagomir (2 μg/μL) was mixed with lipofectamin (1 μL), incubated at room temperature for 30 min, and then intraperitoneal injection was performed on treated group zebrafish with a microsyringe. The semen is artificially squeezed out before each injection to promote the spermatogenesis process. The mixture was injected once a week, every 4 weeks for a treatment cycle, each fish was injected with 6 μL, and a total of 15 zebrafish were injected. The negative control group was injected with a mixture containing negative control antagomir (Negative control antagomir: 5'-CAG UAC UUU UGU GUA GUA CAA-3′). Antagomir treatment methods refer to the published papers [26].

2.9. Histology of gonad

Gonadal tissue surgically removed from treated zebrafish were fixed in Bouin's solution. The fixed tissues were dehydrated and embedded in paraffin. Sections were cut at 5–6 μm using a Leica RM2015 Microtome (Leica, Germany), then transferred to slides, which were processed for haematoxylin and eosin staining. The procedure followed the method described in our published paper [27].

2.10. Morphological observation of sperm

Semen was collected from treated zebrafish, and fixed with 2.5% glutaraldehyde for 2 h. Part of the fixed semen was evenly coated on the cover glass, dried at room temperature, dehydrated with 50%, 70%,

Table 1
Statistical and comparative efficiency of sequencing output data.

Sample	ID	Raw_reads	Clean_reads	Q30(%)
Diploid testis	S01	15,456,966	11,993,451	99.2
	S02	18,098,031	14,145,771	99.07
	S03	26,117,550	21,237,464	99.09
Triploid testis	S04	13,244,069	10,780,393	99.17
	S05	19,712,942	15,447,197	99.12
	S06	20,810,487	15,350,070	99.23

80%, 90%, 95%, 100% alcohol gradient, 10–15 min each time, then freeze drying by freeze dryer (VD-250R, Japan) and observed by scanning electron microscope (Jsm-6360, Japan). The long diameter and short diameter of 50 sperm heads were measured on the picture. The sperm head volume is calculated using the formula (4/3)πabˆ2, where "a" represents the long radius and "b" represents the short radius.

2.11. Fertility assessment

Treated male zebrafish and normal female zebrafish were artificially spawning: male to female was 1:1, they were put into the box and treated in the dark for 10–14 h. Then, the fish was irradiated under a lamp (40 W) at a distance of 10-20 cm, the eggs were laid by themselves, and the fertilization rate of the embryos was calculated under the microscope after 2 h development. 0.5 μL semen from the treated (TD), negative control (NC) and wild-type (WT) male zebrafish were collected with a pipette (0–2.5 μL) and diluted in 1 mL Hank's solution. The density and DNA content of the sperm were detected by flow cytometry, The procedure followed the method described in our published paper [22]. Three biological replicates were performed for all experiments.

2.12. Stastic analysis

The significant differences between the compared groups of samples were analyzed by independent samples *t*-tests in this paper.

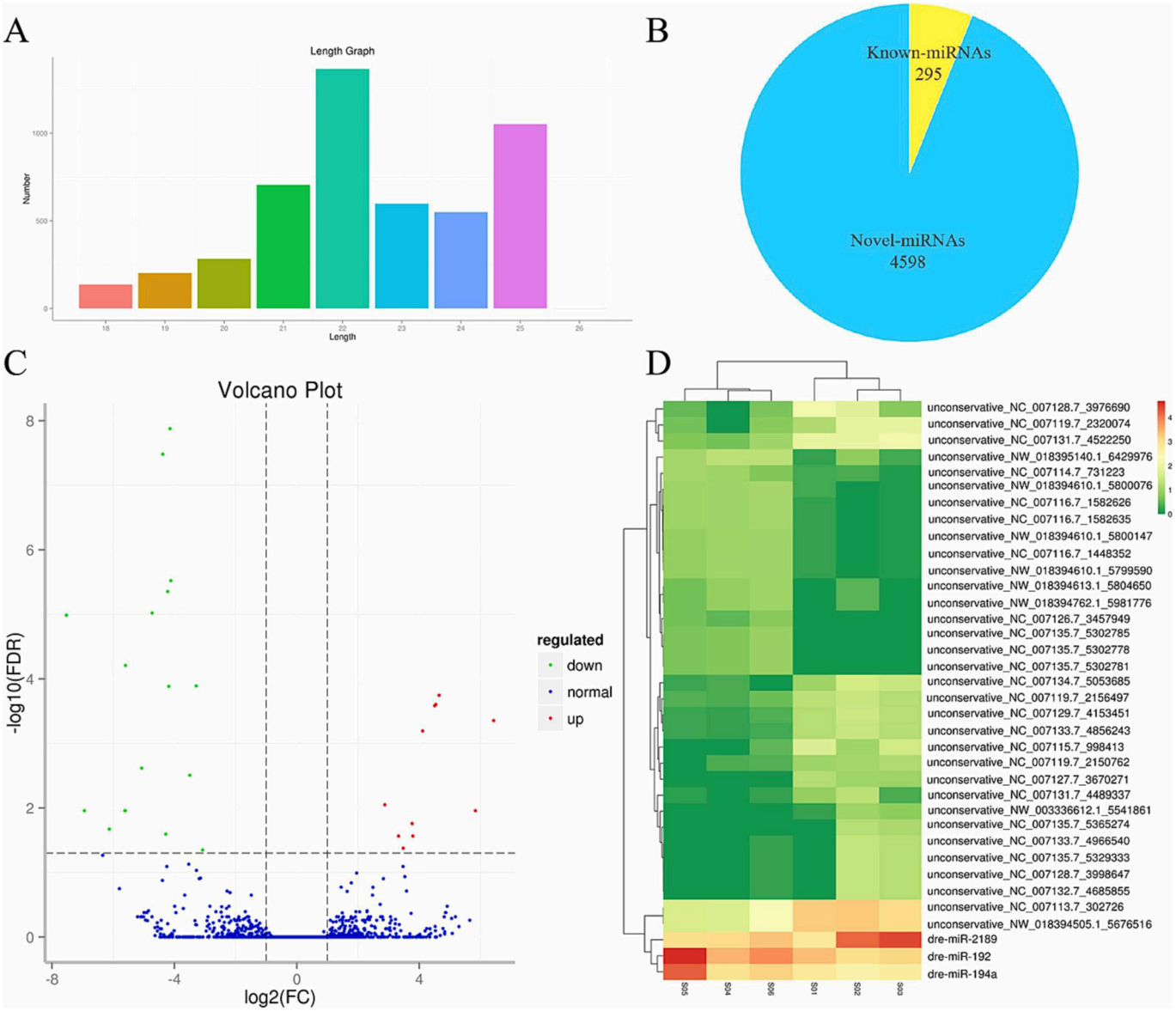

Fig. 1. Transcriptomic analysismature testis of triploid and diploid zebrafish. (A) Length distribution of miRNAs. (B) Results of miRNAs identification. (C) Volcano map of differentially expressed miRNAs. (D) Differentially expressed miRNAs cluster.

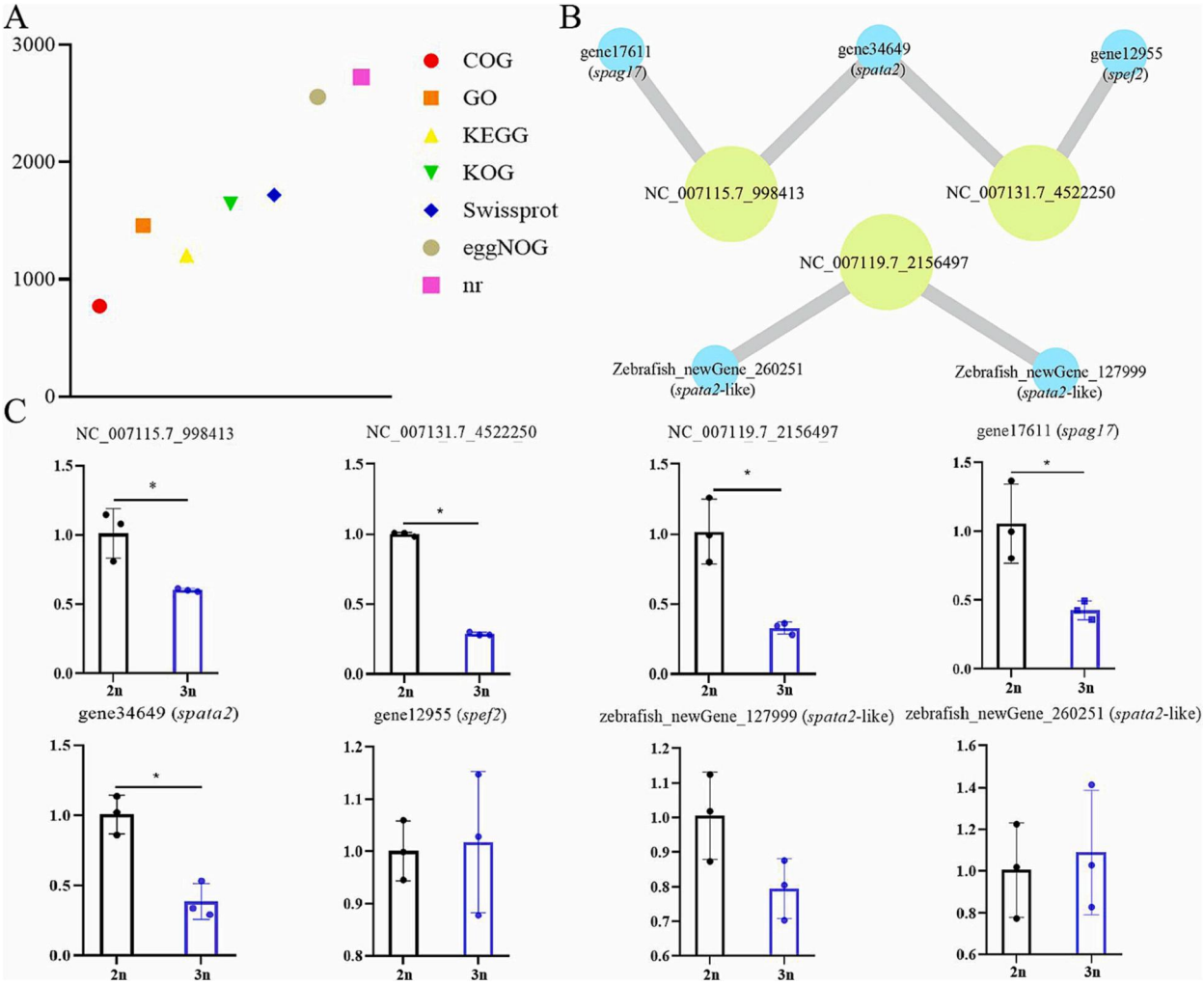

Fig. 2. Analysis of differentially expressed miRNAs and their target genes. (A) Statistics of functional annotation of target genes. (B) The relationship between miRNAs and target genes regulated by male reproductive development. (C) Expression analysis of NC_007115.7_998413, NC_007131.7_4522250, NC_00711 9.7_2156497 and their target genes, *: $P < 0.05$.

3. Results

3.1. MicroRNA expression profile analysis of mature testis in triploid zebrafish

A total of 206.22 M high-quality short-chain RNA data was obtained, with each sample containing 10.78 M of clean data (Table 1). Through alignment and gene structure optimization analysis, 4893 mature miRNAs were detected, whose lengths were concentrated in the range of 20 nt to 26 nt, mainly 22 nt (Fig. 1A). Furthermore, by comparing with the mature miRNA sequences in miRNA database miRBase (v21), 295 known-miRNAs and 4598 novel-miRNAs were obtained (Fig. 1B). Using DESeq R package (1.10.1) to analyze the differential expression and clustering of miRNAs, 36 differentially expressed miRNAs in the triploid zebrafish, including 3 known-miRNAs and 33 novel-miRNAs. Among them, 16 miRNAs (including 2 known-miRNAs) were up-regulated, and 20 miRNAs (including 1 known-miRNAs) were down-regulated (Fig. 1C, D).

3.2. Annotated target genes of differentially expressed miRNA to testicular development

Base on the sequencing data and genome sequence information of zebrafish, target genes were predicted using miRanda and RNAhybrid. A total of 2737 predicted target genes were predicted from 36 differentially expressed miRNAs, of which 2725 were annotated by Nr database, 1206 by KEGG database, and 1459 by GO database (Fig. 2A). According to the functional annotation of target genes, 5 target genes were annotated to participate in the regulation of male reproductive development, which were *spata2* (gene34649), *spata2*-like genes (Zebrafish_newGene_260251, Zebrafish_newGene_127 999), *spef2* (gene12955), and *spag17* (gene17611). And founded the 5 predicted target genes were related to 3 miRNAs, named NC_007115.7_998413, NC_007131.7_4522250, and NC_007119.7_2156497. Among them, NC_ 007115.7_ 998413 was associated with *spag17* and *spata2* genes, NC_ 007131.7_ 4522250 was associated with *spata2* and *spef2* genes, while NC_ 007119.7_ 2156497 was associated with the two *spata2*-like genes (Fig. 2B).

We furtherly analyzed the expression of 3 miRNAs and their predicted target genes in testis of triploid and diploid zebrafish. The expression levels of *spag17*, *spata2* and 3 miRNAs (NC_007115.7_998413, NC_007131.7_4522250, NC_007119.7_2156497) in the triploid zebrafish were significantly down-regulated than those of diploid ($P < 0.05$), while there was not significantly different in those of two *spata2*-like genes and *spef2* ($P > 0.05$) (Fig. 2C). The above results indicated that NC_007115.7_998413 and NC_007131.7_4522250 might be the candidate miRNAs associated with testicular development, rather than NC_007119.7_2156497.

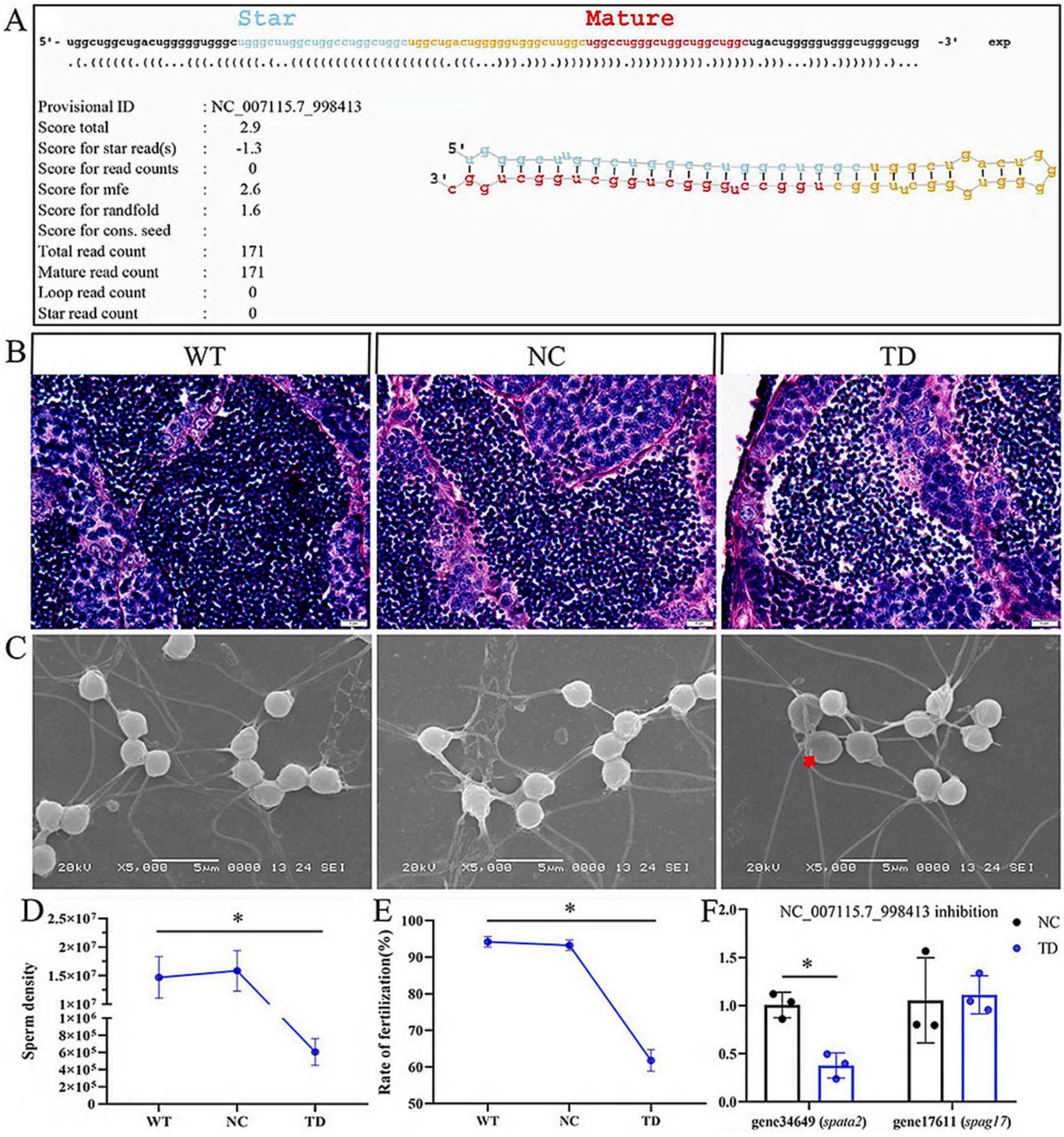

Fig. 3. Effect of inhibiting the activity of NC_007115.7_998413 on testis of zebrafish. (A) Secondary structure prediction of precursors, mature sequence (red), ring structure (yellow) and star sequence (blue). (B, C) Observation of testis histology and sperm morphology of wild type (WT), negative control (NC) and treated (TD) zebrafish. Red arrow indicates sperm with enlarged head. (D, E) Statistics of sperm density and fertilization rate. (F) qRT-PCR verification *: $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Effect of inhibiting the activity of candidate miRNAs on fertility of male zebrafish

Combined with the sequencing results, we obtained the precursor sequences of NC_007115.7_998413 and NC_007131.7_4522250 with the sequence lengths of 115 nt and 112 nt, respectively. Randfold tool was used to predict the secondary structure of the precursor, and the results showed that the precursor of NC_007115.7_998413 had a ring structure with multiple rings, and there was a 22 nt mature sequence at the 3'end of ring structure and a 23 nt star sequence at the 5′ end (Fig. 3A). The precursor of NC_007131.7_4522250 had a ring structure with single rings, and there was a 20 nt mature sequence at the 5'end of ring structure and a 20 nt star sequence at the 3′ end (Fig. S1A).

According to the mature sequences, two special chemically modified miRNA antagonists of NC_007115.7_998413 and NC_007131.7_4522250 were designed to treat male zebrafish *in vivo*. After a 4-

Table 2
Sperm head volume measurement and DNA content detection.

Group	Long diameter (μm)	Short diameter (μm)	Volume ($μm^3$)	Relative DNA content
Wild Type	2.03 ± 0.04	1.94 ± 0.04	3.98 ± 0.20	46.29 ± 1.03
Negative Control	2.03 ± 0.06	1.89 ± 0.08	3.79 ± 0.36	46.27 ± 0.90
Treated	2.36 ± 0.37*	2.18 ± 0.27*	6.16 ± 2.63*	45.95 ± 0.56

* : There are significant differences, $P < 0.05$.

week treatment with the NC_007115.7_998413 or NC_00713 1.7_4522250 inhibitors, the lobular cavity of male zebrafish still maintain mature sperm (Fig. 3B, Fig. S1B). Some abnormal sperm with enlarged head were found in the NC_007115.7_998413 inhibited group (Fig. 3C). And the measurement results showed that volume of abnormal sperm head was 6.16 ± 2.63 $μm^3$, while that of the normal sperm was 3.79 ± 0.36 $μm^3$. Interestingly, flow cytometry analysis showed that there was no change in DNA content of NC_007115.7_998413 inhibited group (Table 2). Additionally, the sperm density in the NC_007115.7_998413 inhibited group (6×10^5 Pcs/μL) was significantly lower than that of the control group (Fig. 3D). Statistical analysis demonstrated a decreased embryo fertilization rate of approximately 65% in NC_007115.7_998413 inhibited group, which was significantly lower than control groups (>90%) (Fig. 3E), but there was no significant difference in the fertilization rate between NC_00713 1.7_4522250 inhibited group and control group (Fig. S1C).

After a 4-week treatment, the expression levels of prediction target genes in miRNA inhibited groups were analyzed by qRT-PCR. The results showed that inhibition of NC_007115.7_998413 significantly downregulated the expression of *spata2*, but did not significantly change the expression of *spag17*. While inhibition of NC_007131.7_4522250, the expression of its predicted target genes (*spata2* and *spef2*) did not change significantly (Fig. 3F, Fig. S1D).

4. Discussion

MiRNA plays important roles in the post-transcriptional regulation of gene expression [28], and changes in miRNA expression were associated with many pathologies [29]. Previous studies have shown that miRNA molecules were potential biomarkers for the diagnosis of spermatogenesis disorders. The expression of *miR-19b* and *Let-7a* in patients with NOA was significantly higher than that in control, but no significant difference was observed in oligospermia patients, and they were also considered to be good molecular biomarkers for the diagnosis of oligospermia [30]. *MiR-192-5p* is preported to have a higher expression level in teratozoospermia and azoospermia patients, its high expression may lead to sperm malformation and reduction, which may be a powerful biomarker for the prognosis of spermatogenesis disorders [31]. In addition, *miR-27a-3p*, *miR-122a* and *miR-10a* have been reported to involved in the regulation of spermatogenesis and affect male reproduction [32–34]. In crucian carp, the higher expression of *miR-199-5p* inhibited the formation of sperm flagella and decreased male fertility [35]. In this research, we performed miRNA transcriptome sequencing on the mature testis of both triploid and diploid zebrafish. Data analysis showed that 36 miRNAs that were differentially expressed in comparison with diploid zebrafish in the testis. At the same time, a key candidate miRNAs, NC_0071157.7.7_998413, was identified by functional annotation and analysis, which are closely associated with the male fertility in zebrafish, and offer a valuable reference for the development of specific markers for spermatogenic disorders.

Spermatogenesis is intricately regulated by an array of genes [36–38]. In mammals, *Spef2* is crucial for sperm tail and cilia function, spontaneous mutation in *spef2* caused male infertility in pig, because of short and immotile sperm tails [39]. *Spag17* encodes a protein present in the active cilia and flagellar axon central pair complex (CPC) that is critical for flagellar assembly and motility, *spag17* mutations lead to sperm anomalies and subsequent male infertility in mice [40,41]. *Spata2*, predominantly expressed in the testicular support cells, showed high expression levels in the testes and brain of humans and rats, unlike other tissues [42,43]. Notably, reduced expression of *spata2* in the testis of men with impaired spermatogenesis implies its significant function in male fertility [43,44]. Furthermore, in adult mice, *spata2* distribution exhibits peak expression in the testes, moderate levels in the epididymis and ovaries, and minimal presence in the brain [45]. *Spata2* knockout in male mice results in lower sperm counts and reduced fecundity [45]. In zebrafish, early *spata2* expression is detectable soon after fertilization, and by 48 h post-fertilization, it localizes to the central nervous system [46]. Remarkably, *spata2* expression persists not only in the testis and brain but also extends to the liver, heart, intestine, muscle, and eye in adult zebrafish [46]. These findings indicate that *spata2* may play a more expansive roles in fish than in mammals. In this research, by functional annotation and miRNA inhibition experiment, we found that the expression level of predicted target gene *spata2* was significantly downregulated upon inhibition of NC_007115.7.7_998413. This finding suggests a potential role for *spata2* in modulating male zebrafish fertility. However, further study was needed to determine whether *spata2* is indeed critical for regulating male fertility in zebrafish.

In conclusion, we performed miRNA transcriptome sequencing on the mature testis of both triploid and diploid zebrafish. Detected 295 known-miRNAs and 4598 novel-miRNAs, from which 36 differentially expressed miRNAs were identified. Furthermore, target genes of differentially expressed miRNAs were predicted, and 2737 target genes were obtained. Subsequent functional annotation and verification of expression levels led to the identification of crucial miRNAs associated with spermatogenesis, including a preliminary investigation into the role of the novel miRNA NC_007115.7_998413 in zebrafish male reproductive development. Our findings shed some light on the intricate regulatory mechanisms of spermatogenesis.

CRediT authorship contribution statement

Wen Fu: Writing – original draft, Formal analysis, Data curation. **Feng Liu:** Formal analysis, Data curation. **Yingying Wang:** Data curation. **Ze Li:** Formal analysis, Data curation. **Wenpei Deng:** Formal analysis. **Wenbin Liu:** Formal analysis, Data curation. **Jinhui Liu:** Formal analysis, Data curation. **Liangyue Peng:** Project administration, Formal analysis. **Yamei Xiao:** Writing – review & editing, Project administration, Funding acquisition, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2024.110813.

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