Current Drug Metabolism, 2019, *20*, 665-673

RESEARCH ARTICLE

Dysregulation of lncRNA and CircRNA Expression in Mouse Testes after Exposure to Triptolide

Suping Xiong[1], Yanting Li[1], Yang Xiang[1], Na Peng[1], Chunmiao Shen[1], Yanqiu Cai[1], Dandan Song[1], Peng Zhang[1], Xiaolong Wang[3], Xuihui Zeng[1] and Xiaoning Zhang[1,2,*]

[1]*Institute of Life Science and School of Life Science, Nanchang University, Nanchang, China;* [2]*Medical School, Institute of Reproductive Medicine, Nantong University, Nantong, China;* [3]*Traditional Chinese Medicine Department, Jilin Women and Children Health Hospital, Changchun, China*

Abstract: ***Background*:** Triptolide has been shown to exert various pharmacological effects on systemic autoimmune diseases and cancers. However, its severe toxicity, especially reproductive toxicity, prevents its widespread clinical use for people with fertility needs. Noncoding RNAs including lncRNAs and circRNAs are novel regulatory molecules that mediate a wide variety of physiological activities; they are crucial for spermatogenesis and their dysregulation might cause male infertility. However, whether they are involved in triptolide-induced reproductive toxicity is completely unknown.

***Methods*:** After exposure of mice to triptolide, the total RNAs were used to investigate lncRNA/circRNA/mRNA expression profiles by strand-specific RNA sequencing at the transcriptome level to help uncover RNA-related mechanisms in triptolide-induced toxicity.

***Results*:** Triptolide significantly decreased testicular weight, damaged testis and sperm morphology, and reduced sperm motility and density. Remarkable deformities in sperm head and tail were also found in triptolide-exposed mice. At the transcriptome level, the triptolide-treated mice exhibited aberrant expression profiles of lncRNAs/circRNAs/mRNAs. Gene Ontology and pathway analyses revealed that the functions of the differentially expressed lncRNA targets, circRNA cognate genes, and mRNAs were closely linked to many processes involved in spermatogenesis. In addition, some lncRNAs/circRNAs were greatly upregulated or inducibly expressed, implying their potential value as candidate markers for triptolide-induced male reproductive toxicity.

***Conclusion*:** This study provides a preliminary database of triptolide-induced transcriptome, promotes understanding of the reproductive toxicity of triptolide, and highlights the need for research on increasing the medical efficacy of triptolide and decreasing its toxicity.

ARTICLE HISTORY

Received: March 02, 2019
Revised: July 05, 2019
Accepted: July 15, 2019

DOI:
10.2174/1389200220666190729130020

Keywords: Triptolide, lncRNA, circRNA, RNA sequencing, spermatogenesis, male infertility.

1. INTRODUCTION

Triptolide is a unique diterpene triepoxide isolated from the Chinese medicinal plant *Tripterygium wilfordii* Hook f., which is used to treat various rheumatological [1] and dermatological conditions [2]. Recently, triptolide has been reported to exert efficient antitumor activity in various human cancers [3-6], and is very promising as a potential new anticancer drug. However, exposure to triptolide could result in injury of various organs in animals and humans [7]. It has also been reported to cause subfertility and infertility by disturbing spermatogenesis and sperm function in rodents [8-10]. These side effects prevent its widespread clinical use for those with fertility needs. There is thus an urgent need to uncover the mechanisms underlying triptolide-induced reproductive toxicity and to identify measures for decreasing triptolide's toxicity.

Long noncoding RNAs (lncRNAs), which are novel regulatory molecules of >200 bp in length, participate in most pathophysiological processes and human diseases. Global genome expression profiles of lncRNAs have indicated that many lncRNAs are highly enriched and exclusively expressed in testes and/or spermatogenic cells [11-13]. Recent studies have also shown that functional deficiency of key lncRNAs could decrease the sperm count in mice, and even cause male infertility in *Drosophila* [14, 15], suggesting that lncRNAs play crucial roles in spermatogenesis. lncRNAs could also act as indicators of stress due to environmental pollutants and boost our understanding of the pharmacological or toxicological mechanisms of drugs and toxicants [16, 17]. However, it has remained unclear whether the abnormal expression and/or regulation of lncRNAs is involved in triptolide-induced infertility.

Circular RNAs (circRNAs) are the products of a unique type of alternative splicing, by which the 3′-end of an exon is spliced to the 5′-end of an upstream exon [18]. The production of circRNAs is probably a highly regulated cell/tissue/age-type-specific process, and among lncRNAs, these molecules are of particular interest in gene regulation. They might thus become biomarkers for diseases of male infertility and exposure to pollutant stress [19, 20]. In a recent report, it was described that key circRNAs participate in testis development or spermatogenesis [21]. Because triptolide could lead to abnormal spermatogenesis, we were interested in whether circRNAs intervened in triptolide-induced reproductive toxicity.

Here, we explored the lncRNA/circRNA-related mechanisms of triptolide-induced male reproductive toxicology by investigating lncRNA/circRNA/mRNA expression profiles at the transcriptome level by strand-specific RNA sequencing.

*Address correspondence to this author at the Institute of Life Science and School of Life Science, Nanchang University, P.O. Box: 330031, Nanchang, China; Tel: +86-791-83827083; Fax: +86-791-83827083;
E-mail: zhangxiaoning1023@outlook.com

1875-5453/19 $58.00+.00

2. MATERIALS AND METHODS

2.1. Chemicals

Triptolide (>98% purity) was purchased from EFEBIO Co., Ltd. (Shanghai, China). All other chemicals obtained from local companies were of analytical purity.

2.2. Animals and Treatments

Ten-week-old male C57BL/6J mice (body weight 25.0 ± 1.5 g) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All mice were kept at constant temperature (22 ± 2 °C) and relative humidity (40%-60%) with a 12/12-h light/dark cycle and were allowed to acclimate for 1 week before the experiments. The mice were divided randomly into two groups. The triptolide group (n = 6) was subjected to the intragastric (i.g.) administration of triptolide at 50 μg/kg body weight/day. The control group (n = 6) was fed saline (0.9% NaCl). The mice were killed by cervical dislocation 35 days after treatment. Testes and epididymal spermatozoa were quickly isolated and harvested for further study. All the experiments were carried out in accordance with the guidelines of the Institutional Animal Ethics Committee (IAEC) of Nanchang University (Nanchang, China).

2.3. Sperm Functional Parameter Analysis

Sperm suspensions were obtained from cauda epididymis and then the sperm were allowed to swim out into high-saline (HS) solution (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM HEPES, 5 mM glucose, 10 mM lactic acid, and 1 mM Na-pyruvate at pH 7.4 with NaOH) in a 5% CO_2/air incubator (Heal Force, Hong Kong, China) at 37 °C for 15 min, as described previously [22]. Sperm concentration, motility, and morphology were analyzed in a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) by a computer-assisted semen analysis system (CASA; Hamilton Thorne, Danvers, MA, USA). At least 200 spermatozoa were counted for each assay.

2.4. Histological Staining

The mouse testes were isolated and immediately fixed in 4% formaldehyde solution, embedded in paraffin, sectioned at a thickness of 5 μm, and then stained with hematoxylin and eosin (H&E). Hepatic pathological changes were observed under a light microscope (Olympus, Tokyo, Japan) at 200× magnification.

2.5. RNA Isolation

Total RNAs were extracted from the testes using TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa, Dalian, China), in accordance with the manufacturer's protocol. The concentration and purity of the total RNA samples were evaluated using a NanoPhotometer (IMPLEN, Munich, Germany) with optical density measurements at A_{260}/A_{280}= 1.8 - 2.2. The integrity of RNA was examined using the 2100 Bioanalyzer (Agilent Corporation, Palo Alto, CA, USA) and by electrophoresis in 1.5% agarose gel to meet sequencing requirements.

2.6. Deep Sequencing and Bioinformatic Analysis

After the rRNA had been depleted from total RNA using Ribo-Zero™ rRNA Removal Kit (Illumina, San Diego, CA, USA), next-generation sequencing library was constructed in accordance with the manufacturer's protocol (NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina®). Sequencing was carried out using a 2 × 150 paired-end (PE) configuration. The sequences were processed and analyzed by GENEWIZ, Inc. (Suzhou, China). After the raw data produced by the Illumina HiSeq™ X Ten platform had been subjected to quality control tests, the clean data were aligned to a reference genome using the software Hisat2 (v2.0.1). Then, HTSeq (v0.6.1) was used to estimate gene and isoform expression levels from the paired-end clean data. Differential expression analysis was performed using the DESeq Bioconductor package, a model based on negative binomial distribution. After adjustment by Benjamini and Hochberg's approach for controlling the false discovery rate, a P-value threshold of <0.05 for genes was set to define differential expressed ones. lncRNA-targeted genes were predicted based on *cis* and *trans* regulatory principles. The *cis* regulation by lncRNAs could be predicted by the software Bedtools (v2.17.0) and regulation in *trans* was analyzed *via* Blast (v 2.2.28+). CIRI (V2.0) software and CircBase were used to identify and screen the circRNAs, and the expression levels of circRNAs were calculated using spliced reads per billion mapping (SRPBM). Gene Ontology (GO; http://www.geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) pathway analyses were applied to determine the functional roles of the differentially expressed lncRNA targets, cognate mRNAs of circRNAs, and mRNAs. GO terms and KEGG pathways with corrected P-values < 0.05 were considered to be significantly associated with the differentially expressed genes.

2.7. Quantitative Polymerase Chain Reaction (qPCR)

One microgram of total RNA was reverse-transcribed to cDNA using PrimeScript™ RT reagent kits with gDNA Eraser (TaKaRa Bio Inc., Otsu, Japan), in accordance with the manufacturer's protocol. The qPCR procedure was performed with denaturation at 98°C for 60 s, followed by 40 cycles of denaturation at 98°C for 5 s, annealing at 58°C for 20 s, and extension at 72°C for 20 s. qPCR was conducted with the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the SYBR Premix DimerEraser Kit (TaKaRa), in accordance with the manufacturer's instructions. The expression level of the *β-actin* gene was used as an internal control to normalize the related gene expression levels. Samples were run at least three times with good reproducibility. The primer sequences for the lncRNAs/circRNAs/mRNAs used for qPCR are shown in Table **1**. All the primers were designed with Primer Premier 5 (Biosoft International, Palo Alto, CA, USA) and produced by GENEWIZ, Inc. (Suzhou, China).

2.8. Statistical Analysis

The data are expressed as the mean ± Standard Error of the Mean (SEM). All the statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Comparisons between the control and triptolide-treated groups were performed using *t*-tests. $P < 0.05$ was regarded as statistically significant and $P < 0.01$ was considered extremely statistically significant.

3. RESULTS

3.1. Effects of Triptolide on Testis and Sperm Morphology and Physiological Sperm Parameters

To confirm triptolide induced reproductive toxicity *in vivo*, histological changes of testes and sperm, and sperm functional parameters were investigated. As shown in Fig. (**1A** and **1B**), triptolide led to significant histological changes in the testis, such as a decrease of spermatogenetic cells, lumen shrinkage, vacuolation, and other morphological abnormalities. After exposure of mice to triptolide for 35 days, there was no significant difference in the body weights of mice in the two groups (Fig. **1C**), the testicular weight decreased significantly ($P < 0.05$) in the triptolide group compared with that in the control group (Fig. **1D**). In addition, there were remarkable increases in sperm head and tail abnormalities (45.75%) (Fig. **1E, 1F** and **1G**) and significant reductions in sperm motility and density after triptolide exposure (Fig. **1H** and **1I**).

3.2. lncRNA, circRNA, and mRNA Expression Profiles

In the present study, upon comparison with the control group, differentially expressed lncRNAs/circRNAs/mRNAs in the testes were detected 35 days after the exposure of mice to triptolide. Our data revealed that there were 33,057 detectable lncRNAs and 11,956 circRNAs in testes. As shown in Fig. (**2**), 344 lncRNAs

Table 1. qPCR primers of examined genes.

Gene ID	Tm(°C)	Length (bp)	Primers (5′→3′)
ENSMUSG00000099722(lnc1)	60	192	F: TCCAGCTTCACCTTGTCACG R: ACGTCTGTTCCGTCATTCCA
ENSMUSG00000106652(lnc2)	60	93	F: GCACAGTGAGAGGAGCATTG R: GATCGGCACCTGATTGGTCT
ENSMUSG00000105299(lnc3)	60	222	F: GGCAACCAGAATCTAGCACT R: GCTGTACATTTTCCTCGGGC
ENSMUSG00000099869(lnc4)	60	118	F: GGAGGGCTGGGATTCACAC R: TCCTCCTCATGCACTTTCACA
ENSMUSG00000021130(lnc5)	60	134	F: GGGTTTAGTGGGGTCAGTCC R: AGGGTGGAAAAGAACGTCCA
ENSMUSG00000092341(lnc6)	60	103	F: TTCAAGGGGCCAGAGAATCC R: CTTCCCAATCCCCACACTGAA
mmu_circ_0000335(cicr1)	60	146	F: GAAGCGACCCAGTCAATCCT R: CATGTGAGCCTCCTCTACGC
mmu_circ_0000947(cicr2)	60	161	F: GTGGTGTTTGCTATGCTGGC R: GAGAAGACCGACCCGTTTGT
Prm1	60	207	F: GCTCACAGGTTGGCTGGCTCG R: TGGTGTATGAGCGGCGGCGA
Prm2	60	337	F: GAGCGCGTAGAGGACTATGG R: CAGACATCGACATGGAATGG
Tnp1	60	81	F: ACCAGCCGCAAGCTAAAGAC R: GCTTCCACCTCTCTTGACGC
Tnp2	60	253	F: CACACCAGTAACCAGTGCAAT R: TAGCTCTGTGAGTCCGTTTCC
β -actin	60	154	F: GGCTGTATTCCCCTCCATCG R: CCAGTTGGTAACAATGCCATGT

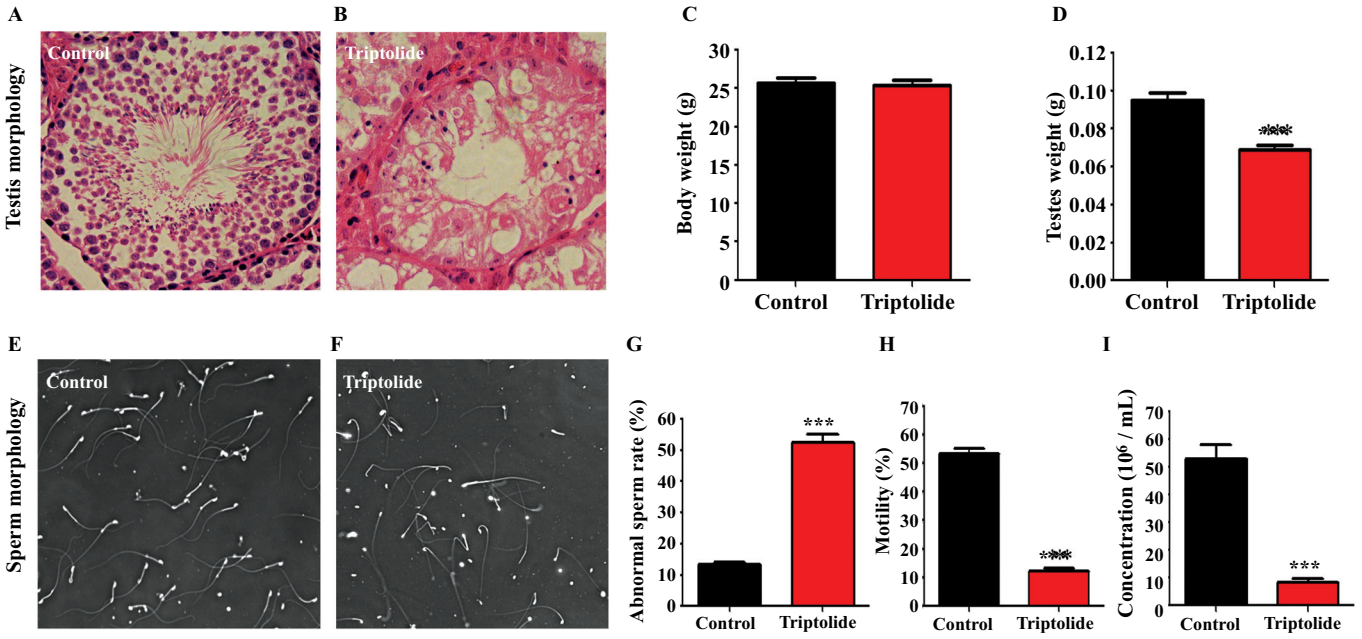

Fig. (1). Effect of triptolide on testis and sperm morphology and physiological sperm parameters. The mice received either physiological saline solution (control) or administered triptolide with i.g. of 50 μg/kg Body weight/day. Mice were euthanized by cervical dislocation at 35 days after treatment. Testes and spermatozoa from the cauda epididymis were harvested quickly. After testis sections were stained with hematoxylin and eosin (H&E), testis morphology (**A**) (**B**) was observed under optical microscope. Body (**C**) and testicular weights (**D**) were measured using an electronic balance. Sperm morphology (**E**) (**F**) was also observed under optical microscope. Sperm abnormality rate (**G**) Sperm motility (**H**), and sperm density (**I**) were analyzed using a CASA system. At least 200 spermatozoa were counted for each assay. Data are presented as the mean ± SE, (n = 6). $^{**}P < 0.01$ compared with control.

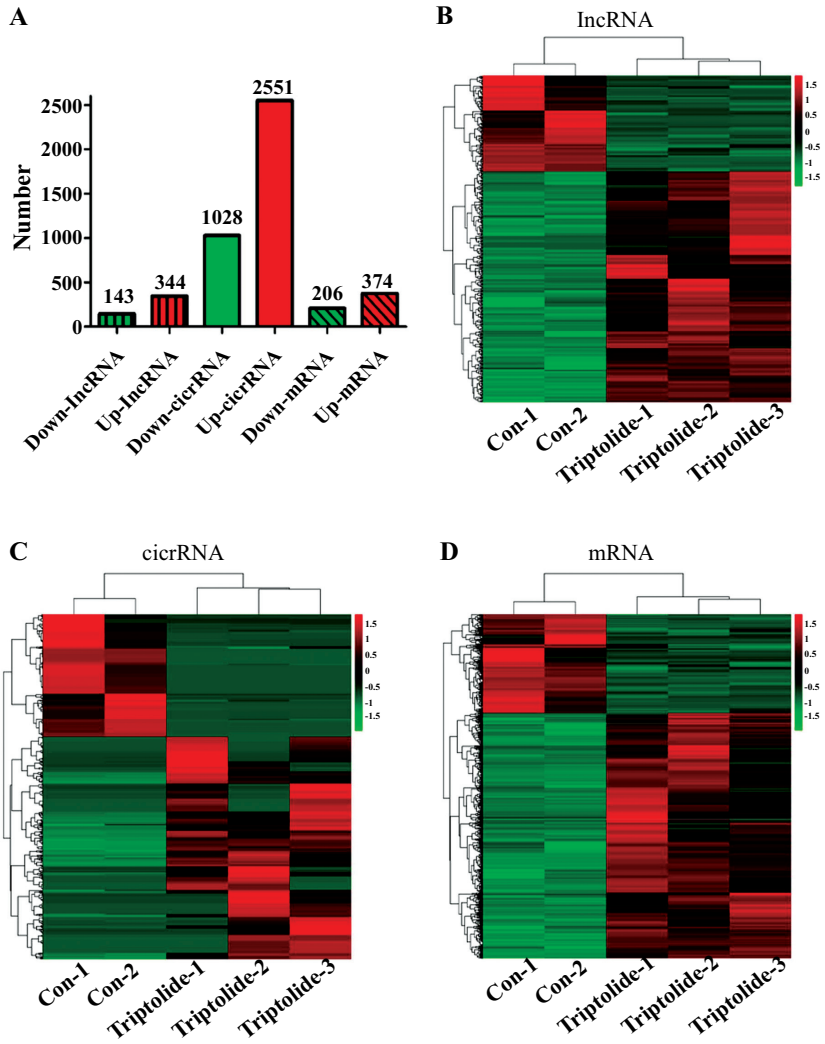

Fig. (2). lncRNA/cicrRNA/mRNA expression profiles after triptolide exposure. The differentially expressed genes were analyzed using DEGseq software based on the FPKM method (≥ 2.0-fold-change with $P < 0.05$). The number of differentially expressed genes (**A**). Differentially expressed lncRNAs (**B**), cicrRNAs (**C**) and mRNAs (**D**) in testes.

were upregulated and 143 were downregulated by triptolide (≥2.0-fold change, $P < 0.05$). For circRNAs, 374 were upregulated and 206 were downregulated after triptolide exposure. Simultaneously, 2551 mRNAs were upregulated and 1,028 were downregulated by triptolide treatment. We speculated that these dysregulated lncRNAs/circRNAs/mRNAs might be good candidate markers for triptolide-induced male reproductive toxicity and would be worthy of further study. Detailed information on all of the differentially expressed lncRNAs/circRNAs/mRNAs is provided in Supplementary Tables **S1**-**S3**.

3.3. GO Analysis and KEGG Pathway Analysis

The expression of numerous lncRNAs/circRNAs/mRNAs revealed significant dysregulation after the exposure of mice to triptolide for 35 days. To uncover the functions of abnormal lncRNAs/circRNAs/mRNAs, bioinformatic enrichment analyses of GO terms and KEGG pathways were performed. GO analysis showed that the genes with aberrant lncRNA targets are mainly involved in the following biological processes: genetic imprinting, nodal signaling pathway, regulation of bone mineralization, biomineral tissue development, DNA methylation, DNA alkylation, and RNA metabolic and biosynthetic processes. Differentially expressed circRNA cognate genes mainly participate in post-translational protein targeting endoplasmic reticulum membrane, protein localization to endoplasmic reticulum, biological regulation, nucleoside metabolic process, chromosome separation, chromosome segregation, nuclear division, and organelle fission. The significantly enriched GO terms of the differentially expressed mRNAs included cellular metabolic process, chromosome organization, and DNA metabolic process (Table **2**).

KEGG pathway analysis of the differentially expressed lncRNA target genes identified the main categories of apoptosis, estrogen signaling pathway, GnRH signaling pathway, calcium signaling pathway, cAMP signaling pathway, systemic lupus erythematosus, mismatch repair, autophagy, and PI3K-Akt signaling pathway and other pathways. Analysis of circRNA cognate genes showed that they are involved in the Fanconi anemia pathway, ubiquitin-mediated proteolysis, and thiamine metabolism pathway. Metabolic process, ErbB signaling pathway, actin cytoskeleton, RNA degradation and transport, RNA degradation and transport, apoptosis, cell cycle, and MAPK signaling pathway were affected by triptolide exposure, as shown by mapping the differentially expressed mRNA genes to the KEGG database (Table **3**).

3.4. qPCR Confirmation

We randomly selected six lncRNAs and two circRNAs from the pool of lncRNAs/circRNAs with fold change > 2.0 and analyzed their expression levels by a qPCR in testes to validate the reliability

Table 2. Top 10 GO terms of differently expression genes.

lncRNA Targets	CicrRNA Cognate Genes	mRNAs
Genetic imprinting	Posttranslational protein	Cellular metabolic process
Regulation of bone mineralization	Biological regulation	Macromolecule metabolic process
DNA methylation involved in embryo development	Nuclear division	Macromolecule metabolic process
Syncytium formation	Chromosome separation	Chromosome organization
Bio-mineral tissue development	Nuclear chromosome segregation	DNA metabolic process
Gene expression by genetic imprinting	Nucleoside metabolic process	Nitrogen compound metabolic
DNA methylation/demethylation	Nuclear division	Aromatic compound metabolic
Regulation of ossification	Organelle fission	Heterocycle metabolic process
Nodal signaling pathway	Ras protein signal transduction	Macromolecule modification
RNA biosynthetic/metabolic process	Protein K63-linked ubiquitination	Protein metabolic process

Table 3. Top 10 KEGG pathways of differently expression genes.

lncRNA Targets	CicrRNA Cognate Genes	mRNAs
Apoptosis	Thiamine metabolism	Metabolic process
Estrogen signaling pathway	Sulfur relay system	ErbB signaling pathway
GnRH signaling pathway	Fanconi anemia pathway	Actin cytoskeleton
Calcium signaling pathway	Ubiquitin mediated proteolysis	RNA degradation and transport
cAMP signaling pathway	-	Apoptosis
Systemic lupus erythematosus	-	Systemic lupus erythematosus
Mismatch repair	-	GnRH signaling pathway
Autophagy	-	Ribosomal pathways
PI3K-Akt signaling pathway	-	Cell cycle
Cytokine-cytokine receptor interaction	-	MAPK signaling pathway

of our sequencing data. We also further investigated the expression levels of four spermatogenesis-required mRNA genes (Prm1, Prm2, Tnp1, and Tnp2), which were selected from 54 differentially expressed mRNAs enriched for the spermatogenesis GO term (Table **4**). As shown in Fig. (**3**), the qPCR results were consistent with the sequencing data and showed the same trend of dysregulation for each lncRNA/circRNA/mRNA (Fig. **3**).

4. DISCUSSION

In a clinical context, *T. wilfordii* extract has been shown to result in impaired fertility in men [23]. One of its active elements, triptolide, was found to exert a powerful sterilization effect for males and was tested in animal studies as a candidate male contraceptive agent [10]. This has prevented triptolide from being widely used to treat immune system diseases and being developed as an anticancer drug for those who wish to have children. Therefore, in the present study, our aim is to explore the mechanisms of triptolide-induced reproductive toxicity using mice as a model for analyses at the global transcriptome level. Our results revealed that testis weight and morphology in the triptolide-treated group were notably different from those in the control group. Furthermore, sperm motility and density in the cauda epididymis were dramatically impaired 35 days after treating mice with triptolide. Similar results were previously reported in triptolide-exposed rats [24]. Exposure of rats to triptolide from 5 to 70 days was also reported to lead to testis damage and impaired spermatogenesis [8, 9, 25, 26]. These findings suggest that triptolide-induced male infertility is rooted in decreased sperm production, abnormal morphology of the testis and sperm, and dysfunction of mature sperm, such as impaired sperm motility. Following triptolide withdrawal, the recovery of male fertility was relatively slow, suggesting that triptolide damaged not only germ cells in testis but also epididymal sperm [25].

lncRNAs are described as working in a *cis* manner when their effects are restricted to the chromosome from which they are transcribed, while they work in a *trans* manner when they affect genes on other chromosomes [27]. Based on this principle, we obtained 23,064 lncRNA target genes and predicted their functions based on the GO and KEGG databases. The circRNAs can perform similar functions in their cognate genes because the circular and linear RNAs are homologous [28]. The cognate linear RNAs of differentially expressed circRNAs were used to evaluate their potential functions by GO and KEGG analyses. The results showed that dif-

Table 4. Dysregulated spermatogenesis related mRNAs.

Gene ID	Up/Down	Gene ID	Up/Down	Gene ID	Up/Down
Herc2	Up	Ggt1	Up	Herc4	Up
Psme4	Up	Akap9	Up	Tle3	Up
Mlh1	Up	Gsr	Up	Tex19.2	Down
Pax5	Up	Dnmt3a	Up	Tex19.1	Down
Gmcl1	Up	Wipf3	Up	Slc22a16	Down
Asz1	Up	Ttll5	Up	Adam18	Down
Kit	Up	Spata5	Up	Adad1	Down
Sbf1	Up	Arid4a	Up	Ccdc33	Down
Adad1	Up	Tbpl1	Up	Tdrd7	Down
Cdk16	Up	Styx	Up	Txndc8	Down
Slco4c1	Up	Sod1	Up	Hist1h1a	Down
Odf2	Up	Adam18	Up	Gal3st1	Down
Wt1	Up	Gata4	Up	H2afx	Down
Bik	Up	Dnaja1	Up	Tnp2	Down
Arid4b	Up	Ggnbp1	Up	Prm2	Down
Prdx4	Up	Wt1	Up	Ggnbp1	Down
Spag16	Up	Ift81	Up	Tnp1	Down
Tdrd9	Up	Morc1	Up	Prm1	Down

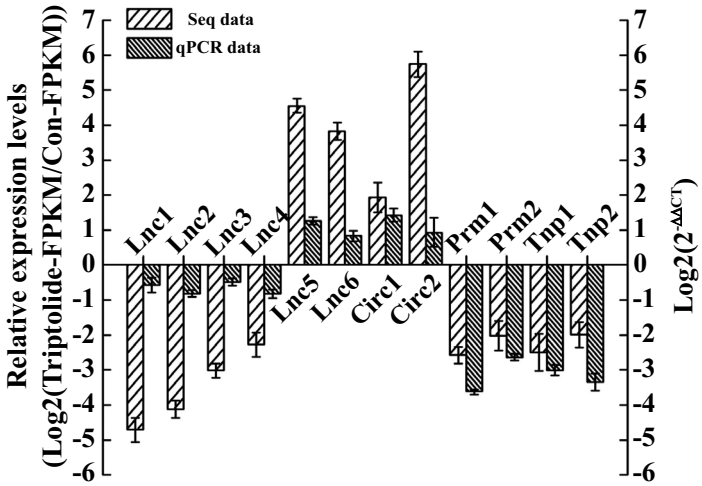

Fig. (3). Validation of sequencing data using qPCR. Total RNA was isolated from testes after exposure of mice to triptolide for 35 days, and then qPCR was performed to detect the RNA expression levels. *β-actin* gene was used as loading control to normalize RNA expression levels. Data are expressed as the mean ± SE ($n = 3$).

ferently expressed lncRNA targets/circRNA cognate genes/mRNAs mainly affected many spermatogenesis-related biological processes and pathways.

Previous studies have suggested that the nodal signaling pathway [29], thiamine metabolism [30], Fanconi anemia pathway [31], and DNA methylation [32] are closely related to spermatogenesis. Our GO/KEGG analyses showed that all of the above pathways were disturbed by triptolide-induced differentially expressed genes in testes, implying that these dysregulated pathways might lead to aberrant spermatogenesis after triptolide exposure. In addition, our results indicated that triptolide could serve as a chemical stress factor that appears to block normal meiosis, such as chromosome separation, chromosome segregation, nuclear division, organelle fission, and chromosome organization, thereby contributing to trip-

tolide's reproductive toxicity. All of these processes are indispensable for the correct production of sperm [33, 34], but were disrupted by triptolide. We found that the differentially expressed mRNAs were particularly associated with the actin cytoskeleton as a KEGG pathway. Interestingly, cytoskeletal organization is extremely rigorously controlled and essential for the production of fertile sperm [35]. It can be disturbed by stress factors such as osmotic pressure and other chemicals, resulting in morphological defects, decreased sperm motility, and even aspermatogenesis [36, 37]. Similar to the findings from the present study, data from Wang *et al*. demonstrated that triptolide induced cytoskeletal dysfunction mainly through the dysregulation of actin dynamics and disruption of cell-cell adherens junctions [38].

To ensure that minimum transcriptional and translational activity occurs in mature sperm, sperm RNA is discharged due to loss of most of the cytoplasm. As a result, the level of RNA in sperm is about 1% of that in somatic cells, which indicates that RNA metabolism or turnover plays a considerable role in spermiogenesis and sperm maturation [39]. In our study, RNA metabolism-related GO terms (RNA metabolism and biosynthesis, RNA degradation, and transport) were disturbed by triptolide. It has been reported that triptolide is an inhibitor of RNA polymerase I and II-dependent transcription, leading predominantly to the downregulation of short-lived mRNA [40], which might contribute to RNA turnover. Furthermore, demethylation and de novo methylation of DNA occur and are well controlled during spermatogenesis; aberrant alterations in DNA methylation could induce abnormal male reproductive performance including infertility [41, 42]. Here, lncRNA targets that were differentially expressed due to triptolide struck a balance of DNA methylation levels, suggesting that an epigenetic mechanism may be involved in triptolide-induced reproductive toxicity. It is worth mentioning that genetic imprinting was also reported to be affected by triptolide, which might result from changes in DNA methylation since DNA methylation is pivotal in the process of imprinting [43]. Moreover, abnormal morphological features in the testis, such as a decrease of spermatogenetic cells, lumen shrinkage, vacuolation, and a decreased number of sperm, provided evidence of the disruption of apoptosis, autophagy, and the cell cycle. Indeed, it has been reported that triptolide-induced toxicity acts on apoptosis, autophagy, and the cell cycle [7].

Previous studies have reported that the cAMP/PKA pathway participates in the regulation of enzymes involved in estrogen synthesis [44]. Our data also showed that triptolide could disturb the cAMP signaling pathway and reports of triptolide-induced reproductive toxicity revealed that triptolide induced a reduction of cellular cAMP concentration [45, 46], indicating that disruption of the cAMP/PKA pathway contributed to reproductive dysfunction. Taking these findings together, many interacting pathways or biological processes appear to contribute in combination to the male subfertility caused by triptolide.

In the present study, we found the dysregulation of 54 spermatogenesis-related mRNA genes. Many of these genes, such as Tnp1, Tnp2, Prm1, and Prm2, were downregulated. Spermiogenesis is the key step in spermatogenesis, which is strictly regulated and has been proposed to play a vital role in shaping the sperm head. During spermiogenesis, Tnp1 and Tnp2 (transition proteins) appear in step-12 and -13 spermatids, steps in which the histones are displaced by these transition proteins. Mice with Tnp1 and/or Tnp2 deficiency were reported to exhibit teratozoospermia and subfertility, and even infertility [47, 48]. Our data revealed that triptolide decreased the expression levels of both Tnp1 and Tnp2, which might contribute to triptolide-induced sperm deformity, especially in chromatin condensation during spermiogenesis. Next, the transition proteins are replaced by protamines such as Prm1 and Prm2. The presence of both Prm1 and Prm2 is required for proper spermatid maturation and male fertility [49, 50]. Here, triptolide was also shown to disturb protamine expression levels. Taking these findings together, the disorder of crosstalk among all spermatogenesis-related genes could result in triptolide-induced male infertility.

CONCLUSION

In summary, to the best of our knowledge, our study is the first to obtain genome-wide lncRNA/circRNA/mRNA expression profiles in mice with triptolide-induced subfertility, which was achieved using strand-specific RNA sequencing in testes. This study provides a solid theoretical foundation and preliminary database for further research into the molecular mechanisms by which lncRNAs/circRNAs play significant roles in triptolide-induced reproductive toxicity. Additionally, mice with triptolide-induced infertility might serve as a good model for revealing the functional noncoding RNAs involved in spermatogenesis.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All the experiments were carried out in accordance with the guidelines of the Institutional Animal Ethics Committee (IAEC) of Nanchang University (Nanchang, China).

HUMAN AND ANIMAL RIGHTS

No humans were involved in this study, the reported experiments on animals were in accordance with the standards set forth in the 8th Edition of Guide for the Care and Use of Laboratory Animals (http:// grants.nih.gov/grants/olaw/Guide-for-thecare-and-use-of-laboratory-animals.pdf) published by the National Academy of Sciences.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The RNA-seq data sets generated in this study have been submitted to the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) under accession number GSE126857.

FUNDING

This research was supported by the National Natural Science Foundation of China (81760283 and 31801238).

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

We thank Emily Crow, PhD, from Liwen Bianji, Edanz Editing China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

REFERENCES

[1] Wang, X.; Zu, Y.; Huang, L.; Yu, J.; Zhao, H.; Wen, C.; Chen, Z.; Xu, Z. Treatment of rheumatoid arthritis with combination of methotrexate and Tripterygium wilfordii: A meta-analysis. *Life Sci.,* **2017**, *171*, 45-50. [http://dx.doi.org/10.1016/j.lfs.2017.01.004] [PMID: 28088452]

[2] Li, C.; Sun, X.; Cao, Y.; Xu, W.; Zhang, W.; Dong, Z. Case report: Remarkable remission of SAPHO syndrome in response to *Tripterygium wilfordii* hook f treatment. *Medicine,* **2017**, *96*(47), e8903 [http://dx.doi.org/10.1097/MD.0000000000008903] [PMID: 29382023]

[3] Kim, S.T.; Kim, S.Y.; Lee, J.; Kim, K.; Park, S.H.; Park, Y.S.; Lim, H.Y.; Kang, W.K.; Park, J.O. Triptolide as a novel agent in pancreatic cancer: The validation using patient derived pancreatic tumor cell line. *BMC Cancer,* **2018**, *18*(1), 1103.

[http://dx.doi.org/10.1186/s12885-018-4995-0] [PMID: 30419860]
[4] Yang, Y.; Zhang, L.J.; Bai, X.G.; Xu, H.J.; Jin, Z.L.; Ding, M. Synergistic antitumour effects of triptolide plus gemcitabine in bladder cancer. *Biomed. Pharmacother.,* **2018**, *106*, 1307-1316. [http://dx.doi.org/10.1016/j.biopha.2018.07.083] [PMID: 30119201]
[5] Yan, P.; Sun, X. Triptolide: A new star for treating human malignancies. *J. Cancer Res. Ther.,* **2018**, *14*(Suppl.), S271-S275. [http://dx.doi.org/10.4103/0973-1482.235340] [PMID: 29970675]
[6] Song, W.; Liu, M.; Wu, J.; Zhai, H.; Chen, Y.; Peng, Z. Preclinical pharmacokinetics of triptolide: A potential antitumor drug. *Curr. Drug Metab.,* **2019**, *20*(2), 147-154. [http://dx.doi.org/10.2174/1389200219666180816141506] [PMID: 30112986]
[7] Xi, C.; Peng, S.; Wu, Z.; Zhou, Q.; Zhou, J. Toxicity of triptolide and the molecular mechanisms involved. *Biomed. Pharmacother.,* **2017**, *90*, 531-541. [http://dx.doi.org/10.1016/j.biopha.2017.04.003] [PMID: 28402922]
[8] Singla, N.; Challana, S. Reproductive toxicity of triptolide in male house rat, *Rattus rattus*. *Sci. World J.,* **2014**, *2014*, 879405. [http://dx.doi.org/10.1155/2014/879405] [PMID: 25374942]
[9] Singla, N.; Kaur, G.; Babbar, B.K.; Sandhu, B.S. Potential of triptolide in reproductive management of the house rat, *Rattus rattus*. *Integr. Zool.,* **2013**, *8*(3), 260-276. [http://dx.doi.org/10.1111/1749-4877.12013] [PMID: 24020465]
[10] Lue, Y.; Sinha Hikim, A.P.; Wang, C.; Leung, A.; Baravarian, S.; Reutrakul, V.; Sangsawan, R.; Chaichana, S.; Swerdloff, R.S. Triptolide: A potential male contraceptive. *J. Androl.,* **1998**, *19*(4), 479-486. [PMID: 9733151]
[11] Luk, A.C.; Gao, H.; Xiao, S.; Liao, J.; Wang, D.; Tu, J.; Rennert, O.M.; Chan, W.Y.; Lee, T.L. GermlncRNA: A unique catalogue of long non-coding RNAs and associated regulations in male germ cell development. *Database,* **2015**, *2015*, bav044. [http://dx.doi.org/10.1093/database/bav044] [PMID: 25982314]
[12] Necsulea, A.; Soumillon, M.; Warnefors, M.; Liechti, A.; Daish, T.; Zeller, U.; Baker, J.C.; Grützner, F.; Kaessmann, H. The evolution of lncRNA repertoires and expression patterns in tetrapods. *Nature,* **2014**, *505*(7485), 635-640. [http://dx.doi.org/10.1038/nature12943] [PMID: 24463510]
[13] Liang, M.; Li, W.; Tian, H.; Hu, T.; Wang, L.; Lin, Y.; Li, Y.; Huang, H.; Sun, F. Sequential expression of long noncoding RNA as mRNA gene expression in specific stages of mouse spermatogenesis. *Sci. Rep.,* **2014**, *4*, 5966. [http://dx.doi.org/10.1038/srep05966] [PMID: 25097017]
[14] Zhang, C.; Gao, L.; Xu, E.Y. LncRNA, a new component of expanding RNA-protein regulatory network important for animal sperm development. *Semin. Cell Dev. Biol.,* **2016**, *59*, 110-117. [http://dx.doi.org/10.1016/j.semcdb.2016.06.013] [PMID: 27345292]
[15] Wen, K.; Yang, L.; Xiong, T.; Di, C.; Ma, D.; Wu, M.; Xue, Z.; Zhang, X.; Long, L.; Zhang, W.; Zhang, J.; Bi, X.; Dai, J.; Zhang, Q.; Lu, Z.J.; Gao, G. Critical roles of long noncoding RNAs in *Drosophila spermatogenesis*. *Genome Res.,* **2016**, *26*(9), 1233-1244. [http://dx.doi.org/10.1101/gr.199547.115] [PMID: 27516619]
[16] Tani, H.; Onuma, Y.; Ito, Y.; Torimura, M. Long non-coding RNAs as surrogate indicators for chemical stress responses in human-induced pluripotent stem cells. *PLoS One,* **2014**, *9*(8), e106282. [http://dx.doi.org/10.1371/journal.pone.0106282] [PMID: 25171338]
[17] Huang, Q.; Liu, Y.; Dong, S. Emerging roles of long non-coding RNAs in the toxicology of environmental chemicals. *J. Appl. Toxicol.,* **2018**, *38*(7), 934-943. [http://dx.doi.org/10.1002/jat.3595] [PMID: 29388697]
[18] Jeck, W.R.; Sharpless, N.E. Detecting and characterizing circular RNAs. *Nat. Biotechnol.,* **2014**, *32*(5), 453-461. [http://dx.doi.org/10.1038/nbt.2890] [PMID: 24811520]
[19] Dong, W.W.; Li, H.M.; Qing, X.R.; Huang, D.H.; Li, H.G. Identification and characterization of human testis derived circular RNAs and their existence in seminal plasma. *Sci. Rep.,* **2016**, *6*, 39080. [http://dx.doi.org/10.1038/srep39080] [PMID: 27958373]
[20] Sai, L.; Li, L.; Hu, C.; Qu, B.; Guo, Q.; Jia, Q.; Zhang, Y.; Bo, C.; Li, X.; Shao, H.; Ng, J.C.; Peng, C. Identification of circular RNAs and their alterations involved in developing male *Xenopus laevis* chronically exposed to atrazine. *Chemosphere,* **2018**, *200*, 295-301. [http://dx.doi.org/10.1016/j.chemosphere.2018.02.140] [PMID: 29494910]
[21] Gao, Y.; Wu, M.; Fan, Y.; Li, S.; Lai, Z.; Huang, Y.; Lan, X.; Lei, C.; Chen, H.; Dang, R. Identification and characterization of circular RNAs in Qinchuan cattle testis. *R. Soc. Open Sci.,* **2018**, *5*(7), 180413. [http://dx.doi.org/10.1098/rsos.180413] [PMID: 30109096]
[22] Zhang, X.; Gao, F.; Fu, J.; Zhang, P.; Wang, Y.; Zeng, X. Systematic identification and characterization of long non-coding RNAs in mouse mature sperm. *PLoS One,* **2017**, *12*(3), e0173402. [http://dx.doi.org/10.1371/journal.pone.0173402] [PMID: 28291811]
[23] Qian, S.Z. *Tripterygium wilfordii*, a Chinese herb effective in male fertility regulation. *Contraception,* **1987**, *36*(3), 335-345. [http://dx.doi.org/10.1016/0010-7824(87)90104-1] [PMID: 3315438]
[24] Huynh, P.N.; Hikim, A.P.; Wang, C.; Stefonovic, K.; Lue, Y.H.; Leung, A.; Atienza, V.; Baravarian, S.; Reutrakul, V.; Swerdloff, R.S. Long-term effects of triptolide on spermatogenesis, epididymal sperm function, and fertility in male rats. *J. Androl.,* **2000**, *21*(5), 689-699. [PMID: 10975416]
[25] Hikim, A.P.; Lue, Y.H.; Wang, C.; Reutrakul, V.; Sangsuwan, R.; Swerdloff, R.S. Posttesticular antifertility action of triptolide in the male rat: Evidence for severe impairment of cauda epididymal sperm ultrastructure. *J. Androl.,* **2000**, *21*(3), 431-437. [PMID: 10819451]
[26] Wang, Z.P.; Gu, Z.P.; Cao, L.; Xu, Y.; You, G.D.; Mao, B.Y.; Qian, S.Z. Effects of tripchlorolide on the epididymides and testes of rats. *Asian J. Androl.,* **1999**, *1*(3), 121-125. [PMID: 11250778]
[27] Roberts, T.C.; Morris, K.V.; Weinberg, M.S. Perspectives on the mechanism of transcriptional regulation by long non-coding RNAs. *Epigenetics,* **2014**, *9*(1), 13-20. [http://dx.doi.org/10.4161/epi.26700] [PMID: 24149621]
[28] Yang, Q.; Wu, J.; Zhao, J.; Xu, T.; Zhao, Z.; Song, X.; Han, P. Circular RNA expression profiles during the differentiation of mouse neural stem cells. *BMC Syst. Biol.,* **2018**, *12*(Suppl 8), 128. [http://dx.doi.org/10.1186/s12918-018-0651-1] [PMID: 30577840]
[29] Spiller, C.M.; Feng, C.W.; Jackson, A.; Gillis, A.J.; Rolland, A.D.; Looijenga, L.H.; Koopman, P.; Bowles, J. Endogenous nodal signaling regulates germ cell potency during mammalian testis development. *Development,* **2012**, *139*(22), 4123-4132. [http://dx.doi.org/10.1242/dev.083006] [PMID: 23034635]
[30] Fleming, J.C.; Tartaglini, E.; Kawatsuji, R.; Yao, D.; Fujiwara, Y.; Bednarski, J.J.; Fleming, M.D.; Neufeld, E.J. Male infertility and thiamine-dependent erythroid hypoplasia in mice lacking thiamine transporter Slc19a2. *Mol. Genet. Metab.,* **2003**, *80*(1-2), 234-241. [http://dx.doi.org/10.1016/S1096-7192(03)00141-0] [PMID: 14567973]
[31] Jamsai, D.; O'Connor, A.E.; O'Donnell, L.; Lo, J.C.; O'Bryan, M.K. Uncoupling of transcription and translation of *Fanconi anemia* (FANC) complex proteins during spermatogenesis. *Spermatogenesis,* **2014**, *5*(1), e979061. [http://dx.doi.org/10.4161/21565562.2014.979061] [PMID: 26413409]
[32] Sujit, K.M.; Sarkar, S.; Singh, V.; Pandey, R.; Agrawal, N.K.; Trivedi, S.; Singh, K.; Gupta, G.; Rajender, S. Genome-wide differential methylation analyses identifies methylation signatures of male infertility. *Hum. Reprod.,* **2018**, *33*(12), 2256-2267. [http://dx.doi.org/10.1093/humrep/dey319] [PMID: 30358834]
[33] Rathke, C.; Baarends, W.M.; Awe, S.; Renkawitz-Pohl, R. Chromatin dynamics during spermiogenesis. *Biochim. Biophys. Acta,* **2014**, *1839*(3), 155-168. [http://dx.doi.org/10.1016/j.bbagrm.2013.08.004] [PMID: 24091090]
[34] Honda, S.; Hirose, S. Stage-specific enhanced expression of mitochondrial fusion and fission factors during spermatogenesis in rat testis. *Biochem. Biophys. Res. Commun.,* **2003**, *311*(2), 424-432. [http://dx.doi.org/10.1016/j.bbrc.2003.10.008] [PMID: 14592431]
[35] Moreno, R.D.; Palomino, J.; Schatten, G. Assembly of spermatid acrosome depends on microtubule organization during mammalian spermiogenesis. *Dev. Biol.,* **2006**, *293*(1), 218-227. [http://dx.doi.org/10.1016/j.ydbio.2006.02.001] [PMID: 16540102]
[36] Correa, L.M.; Thomas, A.; Meyers, S.A. The macaque sperm actin cytoskeleton reorganizes in response to osmotic stress and contributes to morphological defects and decreased motility. *Biol. Reprod.,* **2007**, *77*(6), 942-953.

[http://dx.doi.org/10.1095/biolreprod.107.060533] [PMID: 17823088]

[37] Gao, Y.; Mruk, D.D.; Lui, W.Y.; Lee, W.M.; Cheng, C.Y. F5-peptide induces aspermatogenesis by disrupting organization of actin- and microtubule-based cytoskeletons in the testis. *Oncotarget,* **2016**, *7*(39), 64203-64220. [http://dx.doi.org/10.18632/oncotarget.11887] [PMID: 27611949]

[38] Wang, X.; Zhao, F.; Lv, Z.M.; Shi, W.Q.; Zhang, L.Y.; Yan, M. Triptolide disrupts the actin-based Sertoli-germ cells adherens junctions by inhibiting Rho GTPases expression. *Toxicol. Appl. Pharmacol.,* **2016**, *310*, 32-40. [http://dx.doi.org/10.1016/j.taap.2016.08.017] [PMID: 27554044]

[39] Johnson, G.D.; Sendler, E.; Lalancette, C.; Hauser, R.; Diamond, M.P.; Krawetz, S.A. Cleavage of rRNA ensures translational cessation in sperm at fertilization. *Mol. Hum. Reprod.,* **2011**, *17*(12), 721-726. [http://dx.doi.org/10.1093/molehr/gar054] [PMID: 21831882]

[40] Vispé, S.; DeVries, L.; Créancier, L.; Besse, J.; Bréand, S.; Hobson, D.J.; Svejstrup, J.Q.; Annereau, J.P.; Cussac, D.; Dumontet, C.; Guilbaud, N.; Barret, J.M.; Bailly, C. Triptolide is an inhibitor of RNA polymerase I and II-dependent transcription leading predominantly to down-regulation of short-lived mRNA. *Mol. Cancer Ther.,* **2009**, *8*(10), 2780-2790. [http://dx.doi.org/10.1158/1535-7163.MCT-09-0549] [PMID: 19808979]

[41] Urdinguio, R.G.; Bayón, G.F.; Dmitrijeva, M.; Toraño, E.G.; Bravo, C.; Fraga, M.F.; Bassas, L.; Larriba, S.; Fernández, A.F. Aberrant DNA methylation patterns of spermatozoa in men with unexplained infertility. *Hum. Reprod.,* **2015**, *30*(5), 1014-1028. [http://dx.doi.org/10.1093/humrep/dev053] [PMID: 25753583]

[42] Marchal, R.; Chicheportiche, A.; Dutrillaux, B.; Bernardino-Sgherri, J. DNA methylation in mouse gametogenesis. *Cytogenet. Genome Res.,* **2004**, *105*(2-4), 316-324. [http://dx.doi.org/10.1159/000078204] [PMID: 15237219]

[43] Elhamamsy, A.R. Role of DNA methylation in imprinting disorders: An updated review. *J. Assist. Reprod. Genet.,* **2017**, *34*(5), 549-562. [http://dx.doi.org/10.1007/s10815-017-0895-5] [PMID: 28281142]

[44] Zhang, D.; Trudeau, V.L. Integration of membrane and nuclear estrogen receptor signaling. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.,* **2006**, *144*(3), 306-315. [http://dx.doi.org/10.1016/j.cbpa.2006.01.025] [PMID: 16516516]

[45] Zhang, J.; Liu, L.; Mu, X.; Jiang, Z.; Zhang, L. Effect of triptolide on estradiol release from cultured rat granulosa cells. *Endocr. J.,* **2012**, *59*(6), 473-481. [http://dx.doi.org/10.1507/endocrj.EJ11-0407] [PMID: 22447140]

[46] Zhang, J.; Jiang, Z.; Mu, X.; Wen, J.; Su, Y.; Zhang, L. Effect of triptolide on progesterone production from cultured rat granulosa cells. *Arzneimittelforschung,* **2012**, *62*(6), 301-306. [http://dx.doi.org/10.1055/s-0032-1309041] [PMID: 22592319]

[47] Adham, I.M.; Nayernia, K.; Burkhardt-Göttges, E.; Topaloglu, O.; Dixkens, C.; Holstein, A.F.; Engel, W. Teratozoospermia in mice lacking the transition protein 2 (Tnp2). *Mol. Hum. Reprod.,* **2001**, *7*(6), 513-520. [http://dx.doi.org/10.1093/molehr/7.6.513] [PMID: 11385107]

[48] Shirley, C.R.; Hayashi, S.; Mounsey, S.; Yanagimachi, R.; Meistrich, M.L. Abnormalities and reduced reproductive potential of sperm from Tnp1- and Tnp2-null double mutant mice. *Biol. Reprod.,* **2004**, *71*(4), 1220-1229. [http://dx.doi.org/10.1095/biolreprod.104.029363] [PMID: 15189834]

[49] Takeda, N.; Yoshinaga, K.; Furushima, K.; Takamune, K.; Li, Z.; Abe, S.; Aizawa, S.; Yamamura, K. Viable offspring obtained from Prm1-deficient sperm in mice. *Sci. Rep.,* **2016**, *6*, 27409. [http://dx.doi.org/10.1038/srep27409] [PMID: 27250771]

[50] Cho, C.; Willis, W.D.; Goulding, E.H.; Jung-Ha, H.; Choi, Y.C.; Hecht, N.B.; Eddy, E.M. Haploinsufficiency of protamine-1 or -2 causes infertility in mice. *Nat. Genet.,* **2001**, *28*(1), 82-86. [http://dx.doi.org/10.1038/ng0501-82] [PMID: 11326282]