

# Overexpression of lncRNA-Gm2044 in spermatogonia impairs spermatogenesis in partial seminiferous tubules

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**ABSTRACT** Long noncoding RNAs (lncRNAs) have been demonstrated to regulate reproduction in mammals. Our previous study revealed that the expression level of lncRNA-Gm2044 was obviously elevated in nonobstructive azoospermia with spermatogonial arrest. Here, a transgenic mouse model of lncRNA-Gm2044 in spermatogonia using the *Stra8* promoter was constructed to explore the roles of upregulated lncRNA-Gm2044 in male fertility. Testicular morphology and fertility weren't affected in transgenic mice expressing lncRNA-Gm2044. However, overexpression of lncRNA-Gm2044 in spermatogonia partially impaired spermatogenesis in the transgenic mice. Then, transcriptome sequencing was executed to find the potential signaling pathway repressing spermatogenesis in germ cells of lncRNA-Gm2044 transgenic mice. Through quantitative analysis of differentially expressed genes, 442 upregulated mRNAs and 147 downregulated mRNAs were displayed in male germ cells of Gm2044-transgenic mice

(**Gm2044-Tg**) compared with non-transgenic mice (**Non-Tg**). Using gene ontology (GO) analysis, differentially expressed genes were shown to play vital roles in RNA\_metabolic\_process, Central\_element, Enzyme\_binding, and Intracellular\_bridge. Using Kyoto encyclopedia of genes and genomes (KEGG) analysis, differentially expressed genes were shown to participate in RNA\_transport, Cell\_cycle, Renin-angiotensin\_system, and Chemokine\_signaling\_pathway. Gene Set Enrichment Analysis (GSEA) revealed that Acrosome\_assembly and Sperm\_plasma\_membrane were involved in the overexpression of lncRNA-Gm2044 blocking spermatogenesis. Furthermore, some of the most differentially expressed mRNAs were verified by RT-qPCR. In addition, we determined that the lncRNA-Gm2044 has no ability to translate into peptides by the bioinformatics method and molecular experiment. Thus, lncRNA-Gm2044 is a novel molecular target for the diagnosis and treatment of male infertility.

**Key words:** testis, spermatogonia, spermatogenesis, lncRNA-Gm2044

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## INTRODUCTION

Abnormal expression of long noncoding RNAs (lncRNAs) and their corresponding pathogenic roles in nonobstructive azoospermia have been illuminated in recent years (Zhou et al., 2019; Sabetian et al., 2021b). miRNA-122-5p promotes human spermatogonia stem cell proliferation and DNA synthesis through regulating the *Cbl* gene and competitively binding to lncRNA-Casc7 (Zhou et al., 2020). Extracellular vesicle lncRNAs

of seminal plasma can be used as biomarkers to decide whether to perform microsurgical sperm extraction for testicular spermatozoa in patients with nonobstructive azoospermia (Xie et al., 2020). LncRNA-linc00467 serves as competing endogenous RNA to influence male gamete generation by affecting the expression levels of the *Lrguk* and *Tdrd6* genes in nonobstructive azoospermia (Bo et al., 2020). Bioinformatics analyses suggest that highly expressed ACE2 in the testes of humans with infertility aggravate reproductive diseases through ACE2-mediated SARS-CoV-2 entry, which may be adjusted by lncRNA signaling pathways (Sabetian et al., 2021a). We previously demonstrated that lncRNA-Gm2044 was significantly elevated in nonobstructive azoospermia with spermatogonial arrest and repressed the proliferation of human testicular cell line NCCIT via working as a host gene for miRNA-202 (Liang et al., 2019). LncRNAs play important roles in human

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spermatogenesis, and their dysexpression can lead to male reproductive disorders.

Gene modified animal models have been widely used in the study of the regulation of reproduction by lncRNAs (Nakagawa et al., 2014; Lewandowski et al., 2020; Hong et al., 2021). Deletion of lncRNA-Teshl results in an abnormal sperm head and a decreased reproductive capacity with an abnormal number of offspring gender through interacting with HSF2 (Hong et al., 2021). The conserved lncRNA locus of Tug1 is indispensable for a normal sperm count and morphology in spermatogenesis, and its knockout can lead to male infertility (Lewandowski et al., 2020). Both mitochondrial aggregation and maternal transcriptome can be affected in lncRNA-Sirena1<sup>-/-</sup> mouse oocytes, although female fertility is normal (Ganesh et al., 2020). LncRNA-Neat1 deficiency randomly causes female mouse infertility due to luteal dysfunction and associated low progesterone (Nakagawa et al., 2014). However, few studies have focused on transgenic mice expressing lncRNA, which explain the roles and molecular mechanisms for abnormal overexpression of lncRNAs in human reproductive tissues.

Classic functional research on lncRNAs includes serving as decoys, recruiters, and scaffolds, which are produced by interactions with proteins and construction of ribosomal nuclear proteins (Senmatsu and Hirata, 2021). Recent studies have demonstrated that lncRNA are able to perform physiological functions in novel and unique ways (Huang et al., 2017; Qian et al., 2021). A small protein peptide can be translated from lncRNA-HOXB-AS3 and inhibit both pyruvate kinase M splicing and glucose metabolism reprogramming, preventing the development of colorectal cancer (Huang et al., 2017). The expression level of lncRNA-LCAT3 was elevated by m6A modification mediated with methyltransferase-like protein 3, which interacts with far upstream element binding protein 1 to promote *Myc* expression and accelerate progression of lung adenocarcinomas (Qian et al., 2021). These explorations on new features of lncRNAs will extend their potential roles in the treatment and prevention of human disease.

To explore the roles of upregulated lncRNA-Gm2044 in male fertility, we constructed a transgenic mouse model of lncRNA-Gm2044 in male germ cells using the *Stra8* promoter. The phenotype and transcriptome sequencing of germ cells were detected in transgenic mice expressing lncRNA-Gm2044. Then, the upregulated and downregulated mRNA presentation, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, Gene Set Enrichment Analysis (GSEA) detection, and RT-qPCR were executed in male germ cells of Gm2044-transgenic mice (Gm2044-Tg) as compared with those of non-transgenic mice (Non-Tg). This study mainly reveals the function and signaling pathway for lncRNA-Gm2044 in male reproduction using transgenic mice of male germ cells overexpressing lncRNA-Gm2044.

## MATERIALS AND METHODS

### Construction of lncRNA-Gm2044 Transgenic Mice

Transgenic mice expressing lncRNA-Gm2044 in male germ cells using the *Stra8* promoter were generated by Cyagen Biosciences (Suzhou), Inc. (Taicang, China) on a C57BL/6N background using the PiggyBac transgenic vector. The genomic DNA was extracted from the tails of newborn mice and genotyped by PCR to identify the mice carrying the desired Gm2044-Tg with the primer (Table S1). Gm2044-Tg mice carrying one transgene were obtained through mating experiments between Tg/+ mice and wild-type mice. The testis and epididymis were collected from the bodies of Gm2044-Tg or Non-Tg mice and stained with hematoxylin-eosin (Servicebio, Wuhan, P. R. China) according to our previous protocol (Hu et al., 2021). Briefly, the tissues of testis and epididymis were embedded in paraffin, cut into 5  $\mu$ m section, stained with hematoxylin-eosin, sealed with neutral gum and observed under microscope. The Ethics Committee for Laboratory Animals of Bengbu Medical College reviewed and approved this research (No. 2018011).

### Transcriptome Sequencing

Male germ cells were isolated from the testes of Gm2044-Tg or Non-Tg mice according to our previous study (Liang et al., 2014; Akintayo et al., 2020). TRIzol (Invitrogen, Carlsbad, CA) was used to isolate RNA from male germ cells by manufacturer's protocol. Each sample of male germ cells was lysed by 1 mL TRIzol, added 0.2 mL chloroform and thoroughly mixed, incubate for 3 min, centrifuged with 15 min at 12,000  $g$  at 4°C. Aqueous phase was transferred into new tube and 0.5 mL isopropanol was added, thoroughly mixed, incubated for 10 min, centrifuged with 10 min at 12,000  $g$  at 4°C. Then, RNA precipitate was washed with 75% ethanol, centrifuged with 5 min at 7,500  $g$  at 4°C, removed supernatant, air-dried for 5 min and dissolved by 50  $\mu$ L RNase-free water. The quality and concentration of RNA were analyzed by agarose electrophoresis and NanoDrop. Then, the rRNA was removed, a library was built and sequenced, and the transcriptional abundance and expression levels were calculated according to our previous study (Hu et al., 2021). GO (Mi et al., 2019; Ashburner, et al., 2000; Gene Ontology Consortium, 2021), KEGG (Kanehisa, 1997; Kanehisa et al., 2021), and GSEA (Mootha et al., 2003; Subramanian et al., 2005) were selected to find the potential downstream molecular pathway of lncRNA-Gm2044 in male germ cells.

### Open Reading Frame Prediction and Plasmid Construction

To determine whether lncRNA-Gm2044 has the ability to be translated into protein peptides, the potential

open reading frame (**ORF**) of lncRNA-Gm2044 was predicted by the ORF Finder (<https://www.ncbi.nlm.nih.gov/orffinder/>; Figure S1). According to a previous study for lncRNA ORF validation (Li, et al., 2020), the complementary DNA (**cdDNA**) of potential ORF and Flag tag were inserted into a pcDNA3.1(+) vector at the EcoR1 and Xho1 restriction sites. The detailed information for the plasmid construction is shown in Table S2. The pcDNA3.1(+) vector was supplied by Dr. Fei Sun (Nantong University, P. R. China). All the constructed plasmids were validated by DNA sequencing.

## Real-Time Quantitative PCR and Western Blotting

The relative expression levels of lncRNA-Gm2044 and mRNAs were detected by real-time Quantitative PCR (**RT-qPCR**). The RNA of male germ cells was reverse transcribed to cDNA by the Evo M-MLV RT Kit (Accurate Biotechnology, Changsha, P. R. China) with RTase Reaction Buffer Mix, Evo M-MLV RTase Enzyme Mix, Oligo dT (18T) Primer and Random 6 mers Primer at 37°C 15 min, 85°C 5 s and 4°C hold. Afterwards, the cDNA was analyzed by RT-qPCR to study lncRNA-Gm2044 and mRNA expression using the SYBR Green Premix Pro Taq HS qPCR Kit (Accurate Biotechnology) at 95°C 30 s, 40 cycles of 95°C 5 s and 60°C 30 s. The primers for lncRNA-Gm2044 and  $\beta$ -actin (internal reference) are listed in our previous research (Hu et al., 2018, 2019), and other primers are shown in Table S3.

The expression level of the target protein was performed by western blotting. The protein was

extracted from cells using the RIPA reagent (Millipore, Bedford, MA) with a protease inhibitor (Roche, Mannheim, Germany), separated with SDS-PAGE, transferred to nitrocellulose membranes, and treated with antibodies of GFP (Thermo Fisher Scientific, Rockford, IL), Flag (Affinity Biosciences, Cincinnati, OH), and  $\beta$ -actin (ABclonal Technology, Wuhan, China).

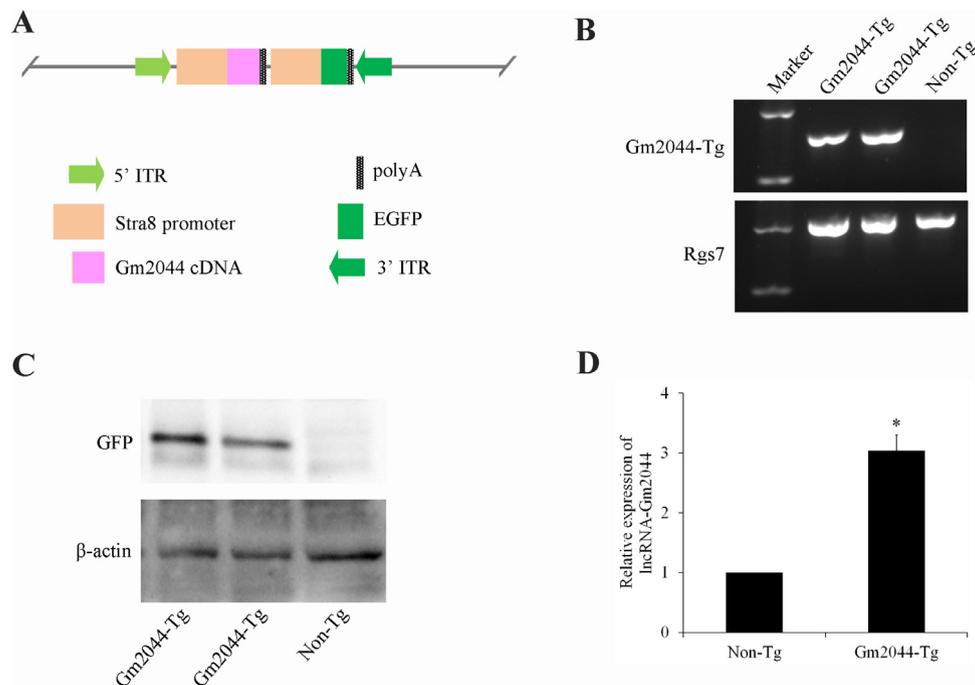
## Statistical Analysis

Data was presented as mean  $\pm$  SEM and analyzed by Independent-Sample *t* Test through SPSS Statistics 21.0. *P* < 0.05 indicated a significant difference.

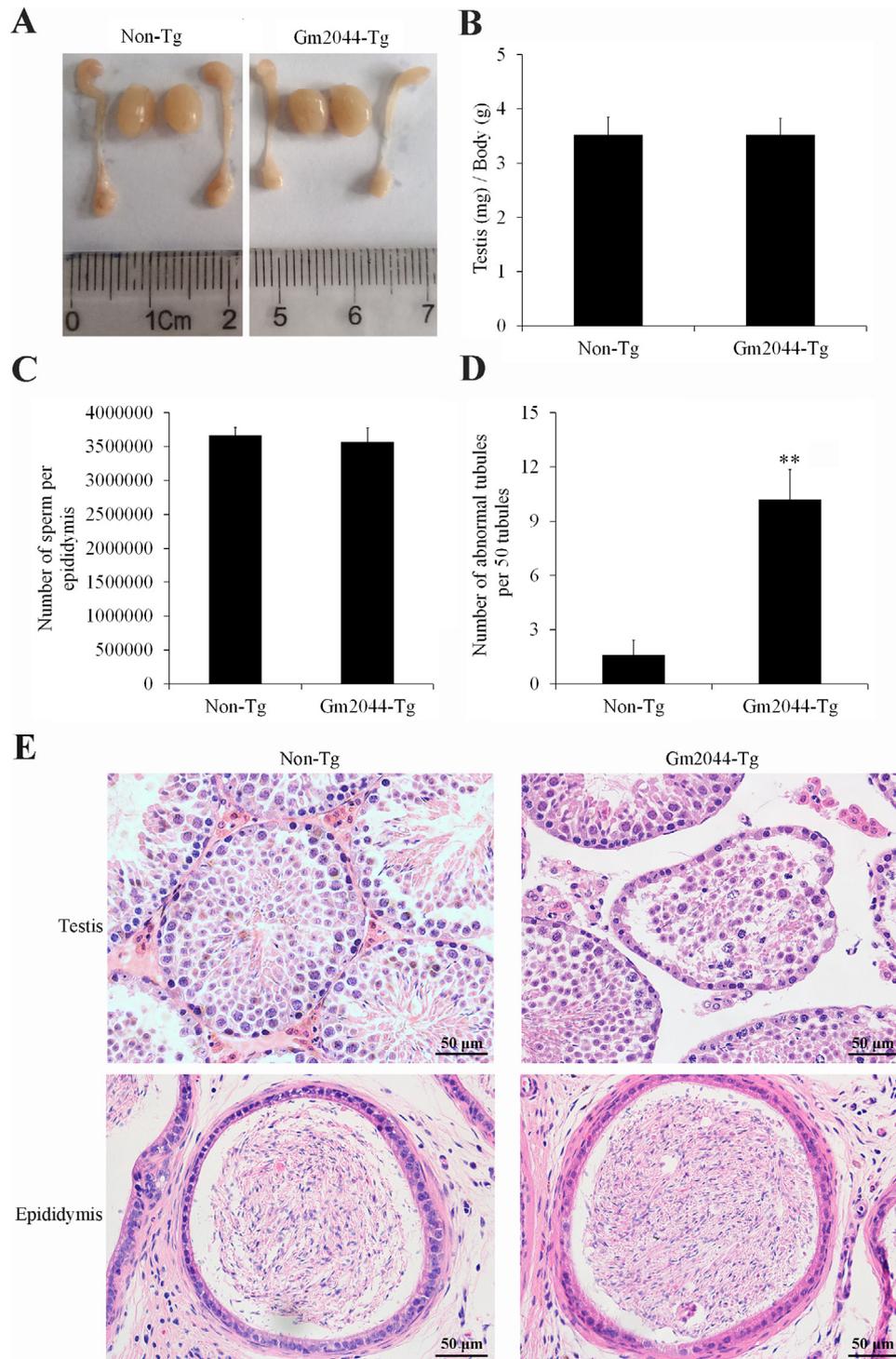
## RESULTS

### Overexpression of lncRNA-Gm2044 Partially Inhibited Spermatogenesis

To determine the effects of upregulated lncRNA-Gm2044 on male fertility, transgenic mice expressing lncRNA-Gm2044 in male germ cells using the *Stra8* promoter were constructed on a C57BL/6N background using the PiggyBac transgenic vector (Figure 1A). The genotype of Gm2044-Tg or non-Tg for transgenic mice was verified by PCR using genomic DNA from the tails of newborn mice (Figure 1B). Furthermore, the effects of overexpression of lncRNA-Gm2044 in Gm2044-Tg mice have been validated by western blotting for GFP (PiggyBac transgenic marker) (Figure 1C) and RT-qPCR for lncRNA-Gm2044 (Figure 1D). The



**Figure 1.** Construction of a transgenic mouse model expressing lncRNA-Gm2044 in male germ cells. (A) Structure sketch of transgenic mice for lncRNA-Gm2044 using the PiggyBac transgenic vector and *Stra8* promoter. (B) Genotyping of transgenic mice for lncRNA-Gm2044. The genomic DNA was extracted from tails of newborn mice and genotyped by PCR to identify the mice carrying the desired Gm2044-Tg. *Rgs7* was used as the reference gene. (C and D) Transgenic mice had significant overexpression of lncRNA-Gm2044. Protein and RNA were isolated from the testes of transgenic mice and subjected to western blotting for the PiggyBac transgenic marker GFP (C) and RT-qPCR for lncRNA-Gm2044 (D).



**Figure 2.** Spermatogenesis analysis of lncRNA-Gm2044 transgenic mice. (A–C) The testicular/epididymal morphology, testicular index, and number of spermatozoa in the epididymis for Gm2044-Tg mice didn't change. The testes and epididymites were separated from Non-Tg and Gm2044-Tg mice and then photographed (A). The value of the testis (mg) divided by the body (g) was calculated for Non-Tg and Gm2044-Tg mice (B). The epididymis was separated from the body of the mouse and then its sperm were released and counted. (D) The number of abnormal seminiferous tubules in Gm2044-Tg mice was five times higher than that in Non-Tg mice. Small, disordered and twisted seminiferous tubules were calculated in the testes of Gm2044-Tg and Non-Tg mice. (E) Overexpression of lncRNA-Gm2044 partially inhibits spermatogenesis. The testes and epididymites were separated from Non-Tg and Gm2044-Tg mice and then stained with hematoxylin-eosin.

testicular/epididymal morphology (Figure 2A), testicular index (Figure 2B), number of spermatozoa in the epididymis (Figure 2C), and male fertility (Table S4) weren't affected in Gm2044-Tg mice as compared with those in non-Tg mice. However, hematoxylin-eosin staining revealed that spermatogenesis was partially

impaired in Gm2044-Tg mice (Figure 2E). For Gm2044-Tg mice, about one-fifth of the seminiferous tubules became small or twisted, and their spermatogenic cells were disordered and reduced in the testis (Figures 2D and 2E). Also, the spermatozoa were not observably changed in the epididymis (Figure 2E).

**Table 1.** RNA quantification and quality assurance.

Sample	OD260/280	OD260/230	Concentration (ng/ $\mu$ L)	Volume ( $\mu$ L)	Quantity (ng)	QC purity (Pass or Fail)
Non-Tg-1	2.04	1.92	1338.40	60	80304.00	Pass
Non-Tg-2	2.01	2.34	1515.70	40	60628.00	Pass
Non-Tg-3	2.02	2.10	1002.97	40	40118.80	Pass
Gm2044-Tg-1	1.93	1.94	258.76	15	3881.40	Pass
Gm2044-Tg-2	1.96	1.96	378.94	15	5684.10	Pass
Gm2044-Tg-3	1.99	2.39	826.98	30	24809.40	Pass

**Table 2.** Statistical table of  $Q$  value for transcripts.

Sample ID	Reads number	Total number of base	Base number ( $Q \geq 30$ )	$Q30$ (%)
Non-Tg-1	45970046	6895506900	6468090637	93.80%
Non-Tg-2	68094690	10214203500	9695828296	94.92%
Non-Tg-3	66867184	10030077600	9499811925	94.71%
Gm2044-Tg-1	52710730	7906609500	7276560135	92.03%
Gm2044-Tg-2	51989136	7798370400	7251647579	92.99%
Gm2044-Tg-3	48606274	7290941100	6842710655	93.85%

### Transcriptome Sequencing for Male Germ Cells in *lncRNA-Gm2044* Transgenic Mice

Male germ cells were isolated from the testes of Gm2044-Tg or Non-Tg mice and then total RNA was extracted. Extracted RNA adhered to the standard for RNA quantification and quality assurance (Table 1). The  $Q30$  value for transcripts was more than 90% (Table 2), and the mapped genome for transcripts was more than 75% (Table 3), which met the standards for transcriptome profiling. Then, the differentially expressed genes were analyzed from transcriptome sequencing data for male germ cells of Gm2044-Tg and non-Tg mice. Principal component analysis showed that 6 samples for Gm2044-Tg and Non-Tg mice had a good clustering effect using significantly differential genes (Figure 3A). As shown with volcano plots (Figure 3B) and heatmaps (Figure 3C), there were 442 significantly upregulated genes and 147 significantly downregulated genes in the Gm2044-Tg germ cells as compared with the non-Tg germ cells, with the fold change  $\geq 1.5$  and  $P \leq 0.05$ . The most number of significantly upregulated genes (46) and significantly downregulated genes (20) were distributed in chromosome X and chromosome 1, respectively (Figure 3D).

### GO, KEGG, and GSEA Analyses for Differentially Expressed Genes

To find the potential downstream molecular pathway of *lncRNA-Gm2044* in male germ cells, differentially

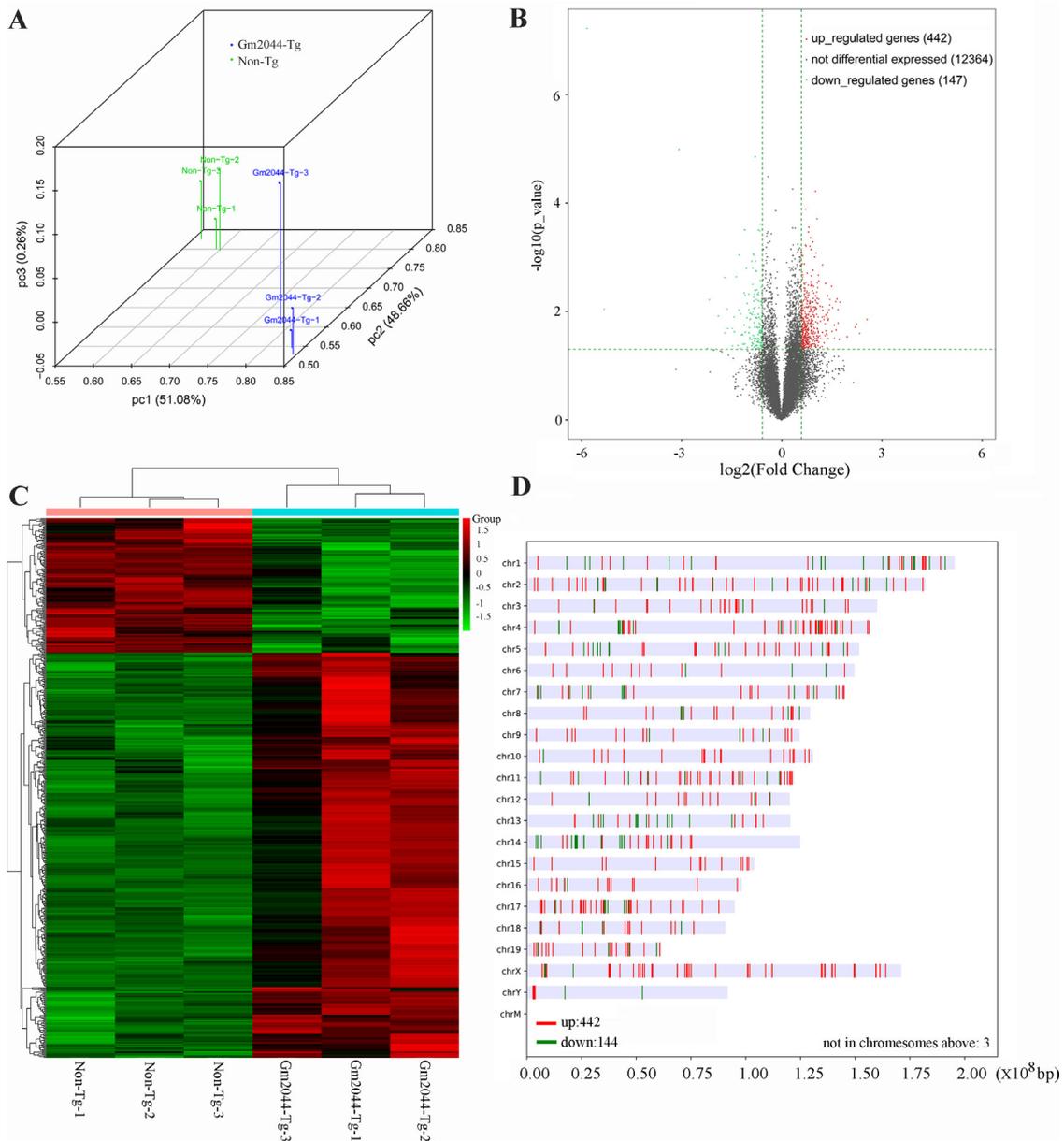
expressed genes were analyzed by the GO, KEGG, and GSEA methods. According to the GO analysis of biological process, cellular component and molecular function for significantly upregulated/downregulated genes, the detailed analysis results are presented in Tables S5–S10. The top term of biological process, cellular component, and molecular function for the GO analysis are shown in Figures 4A–4C (significantly upregulated genes) and Figures 5A–5C (significantly downregulated genes), such as RNA\_metabolic\_process [GO:0016070], Central\_element [GO:0000801], Enzyme\_binding [GO:0019899], Protein\_binding [GO:0005515], Regulation\_of\_renal\_output\_by\_angiotensin [GO:0002019], Intracellular\_bridge [GO:0045171], and Enzyme\_regulator\_activity [GO:0030234].

According to the KEGG analysis for significantly upregulated/downregulated genes, the detailed analysis results are presented in Tables S11 and S12. The top terms for the KEGG analysis are shown in Figure 6A (significantly upregulated genes) and Figure 6B (significantly downregulated genes), such as RNA\_transport [mmu03040] (Figure S2), Cell\_cycle [mmu04110], Renin-angiotensin\_system [mmu04614], and Chemokine\_signaling\_pathway [mmu04062]. GO and KEGG analyses indicate that *lncRNA-Gm2044* may regulate the biological function of RNA during spermatogenesis.

According to the GSEA analysis for differentially expressed gene sets, the detailed analysis results are presented in Tables S13–S16. Representative pathways involved in the reproductive process, Acrosome\_assembly [GO\_GO:0001675] (Figure 6C and 6D) and

**Table 3.** Mapping summary for transcripts.

Sample ID	Raw pairs	Trimmed	mtRNAs	rRNAs	Mapped	Unmapped
Non-Tg-1	22985023	22975558	3.04%	0.18%	91.53%	8.47%
Non-Tg-2	34047345	33812541	5.13%	0.20%	92.60%	7.40%
Non-Tg-3	33433592	32996492	4.94%	0.18%	93.82%	6.18%
Gm2044-Tg-1	26355365	26307358	11.23%	1.26%	77.98%	22.02%
Gm2044-Tg-2	25994568	25966832	4.07%	0.69%	82.76%	17.24%
Gm2044-Tg-3	24303137	24291244	6.14%	0.39%	90.89%	9.11%



**Figure 3.** Transcriptome sequencing for lncRNA-Gm2044 transgenic mice. (A) Significantly differential genes were used to sample clusters with principal component analysis. (B and C) The volcano plots (B) and heatmaps (C) showed significantly upregulated (red) and downregulated (green) genes. (D) The distribution of significantly upregulated genes (red) and significantly downregulated genes (green) in chromosomes.

Sperm\_plasma\_membrane [GO\_GO:0097524] (Figures 6E and 6F) were presented, which suggest that the zona pellucida binding protein (*Zbp*) and A Disintegrin And Metalloproteinase domain (*Adam*) family participates in the regulation of spermatogenesis by lncRNA-Gm2044.

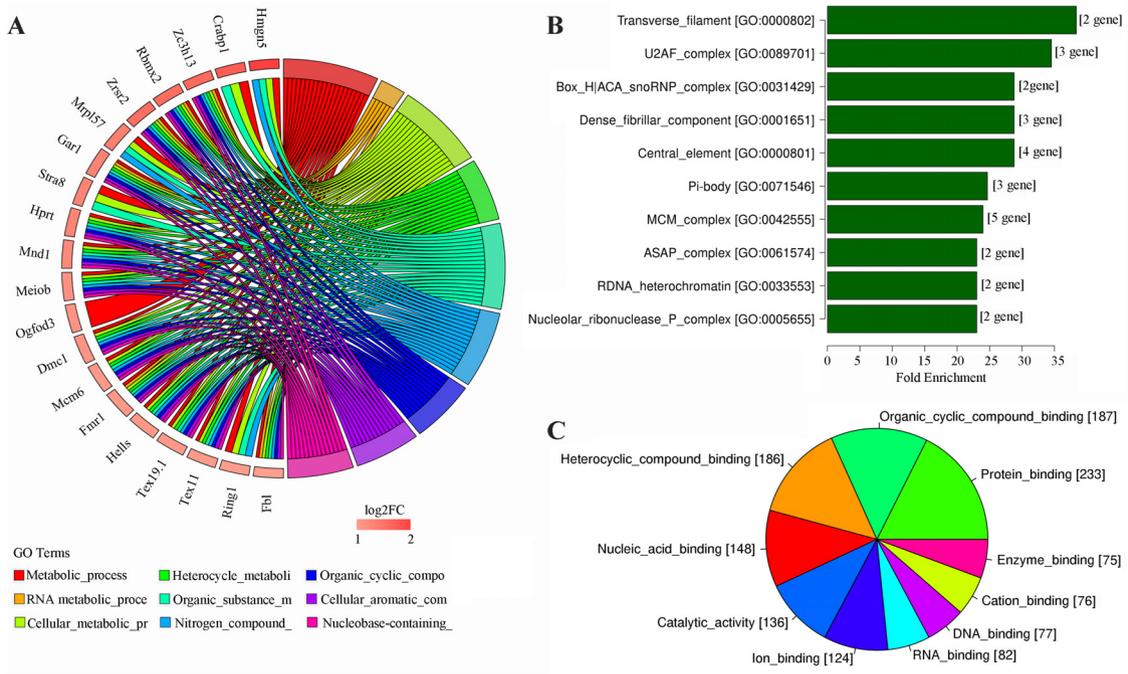
### Validation of Significant Differentially Expressed Genes

The relative expression levels of most significantly upregulated and downregulated genes for transcriptome sequencing data were detected by RT-qPCR. Of the 5 selected significantly upregulated genes (*Ptma*, *Hmgn5*, *Ncl*, *Crabp1*, and *Rhox8*) and 5 selected significantly downregulated genes (*Ccl27b*, *Insl3*, *Lcn2*, *Cyp17a1*, and *Hsd3b1*), only the expression trend of *Rhox8* for RT-

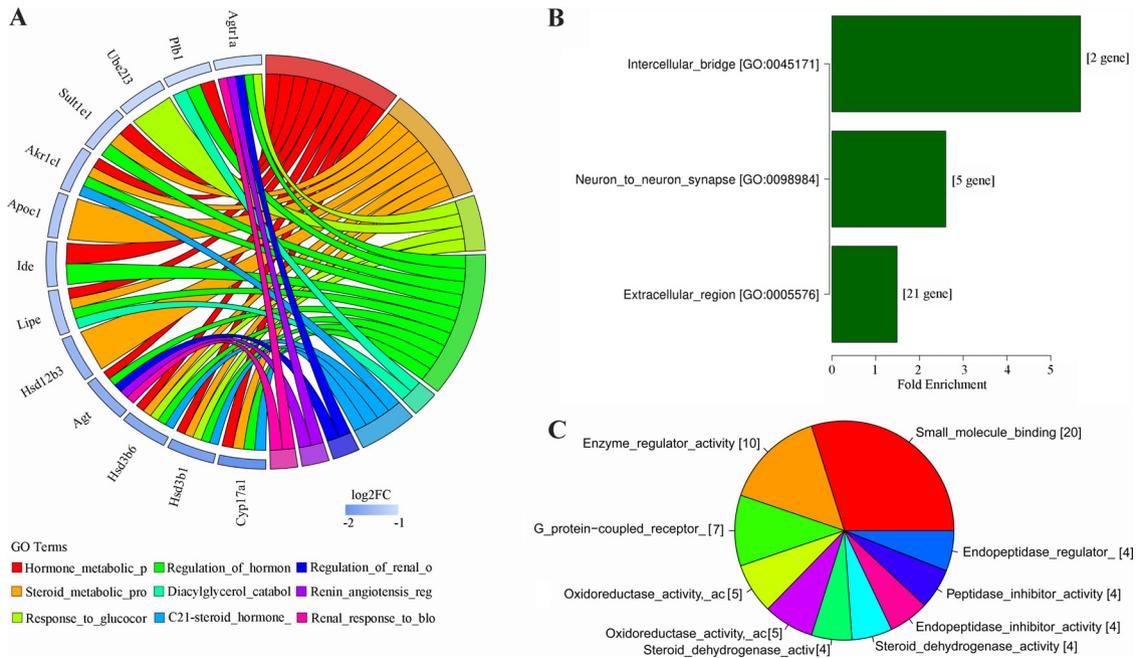
qPCR differed from its transcriptome sequencing (Figure 7A). The expression trends for other genes were consistent between transcriptome sequencing and RT-qPCR (Figure 7A). The transcriptome sequencing data was proven to be highly reliable by RT-qPCR, which provides many potentially important targets for the further study of lncRNA-Gm2044 in male fertility.

### LncRNA-Gm2044 is Not Translated Into Protein Peptides

To verify whether lncRNA-Gm2044 can be translated into protein peptides, the ORF Finder (<https://www.ncbi.nlm.nih.gov/orffinder/>) was used to find the potential ORF for lncRNA-Gm2044. Four potential ORFs were predicted by the ORF Finder, but the potential ORF4 existed in the opposite strand of lncRNA-



**Figure 4.** GO analysis for significantly upregulated genes. (A–C) The top terms of biological process (A), cellular component (B) and molecular function (C) for GO analysis.



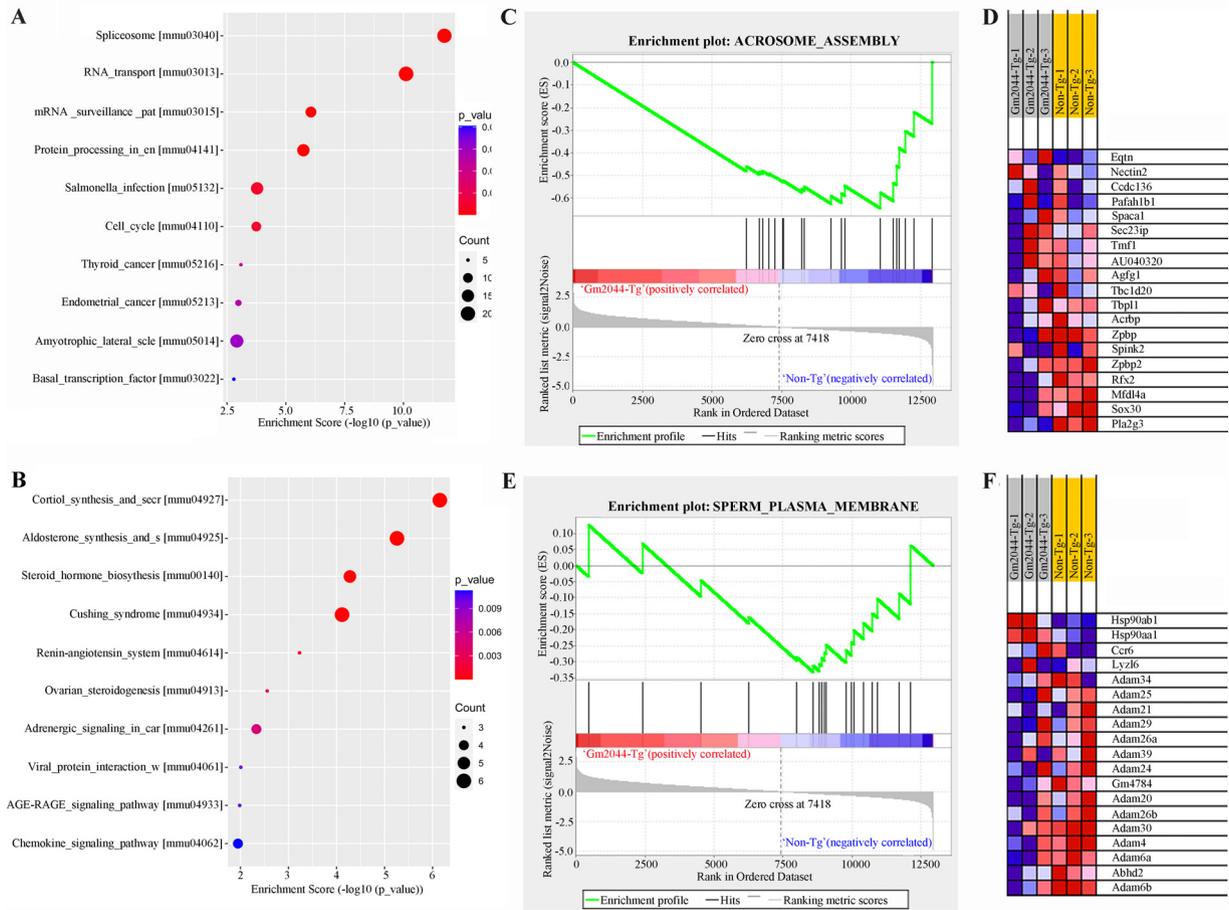
**Figure 5.** GO analysis for significantly downregulated genes. (A–C) The top terms of biological process (A), cellular component (B) and molecular function (C) for GO analysis.

Gm2044 and wasn't able to be translated into protein peptides (Figure S1). The cDNA of potential Gm2044 ORF1/ORF2/ORF3 and Flag tag was inserted after the CMV/T7 promoter in the pcDNA3.1 (+) vector, and Tafa2-Flag was used as the positive control (Figure 7B). The protein was isolated from 293T cells transfected with the pcDNA3.1 (+) vector with potential Gm2044 ORF1/ORF2/ORF3-Flag or Tafa2-Flag and subjected to western blotting. The positive control, Tafa2-Flag, produced a clear protein band, but no protein band was produced by the Gm2044 ORF1/ORF2/ORF3-Flag at

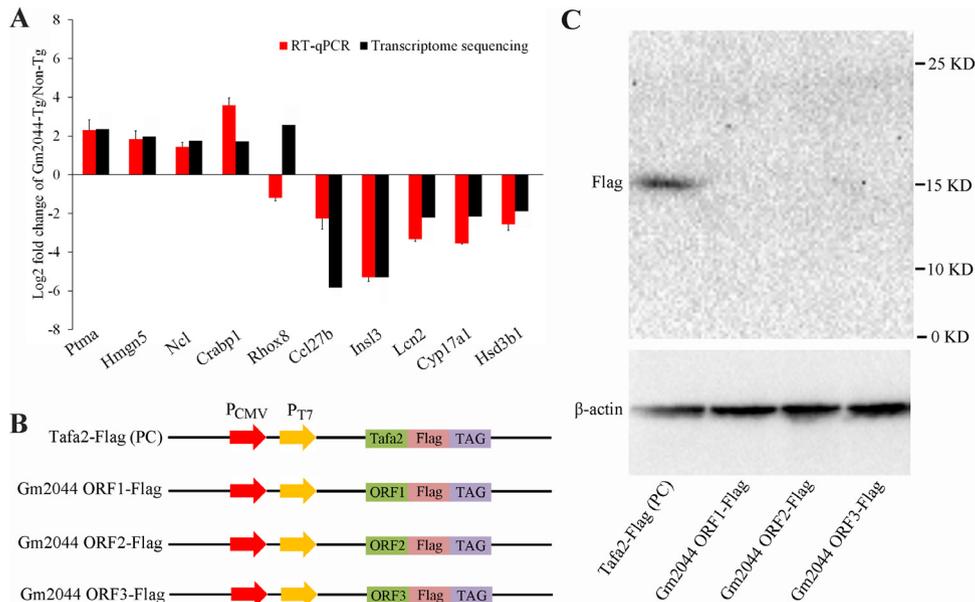
the predicted sites (~5 KD) (Figure 7C). These results prove that lncRNA-Gm2044 does not have the ability to be translated into protein peptides and functions as non-coding RNA.

## DISCUSSION

We previously reported that lncRNA-Gm2044 regulates spermatocyte function through *Sycp1* and *Utf1* under physiological conditions (Hu et al., 2018;



**Figure 6.** KEGG and GSEA analysis for lncRNA-Gm2044 function. (A and B) KEGG analysis for significantly upregulated and downregulated genes. The top terms for KEGG analysis of significantly upregulated genes (A) and significantly downregulated genes (B). (C–F) GSEA analysis for differentially expressed gene sets. The enrichment plot (C) and gene sets (D) of Acrosome\_assembly [GO\_GO:0001675] involved in lncRNA-Gm2044 regulating spermatogenesis. (E and F) The enrichment plot (E) and gene sets (F) of Sperm\_plasma\_membrane [GO\_GO:0097524] involved in the regulation of spermatogenesis by lncRNA-Gm2044.



**Figure 7.** Verification of transcriptome sequencing data and lncRNA-Gm2044 does not have the ability to be translated into protein peptides. (A) Validation of significantly upregulated and downregulated genes in transcriptome sequencing data by RT-qPCR. Log<sub>2</sub> fold change of Gm2044-Tg/Non-Tg was analyzed to compare the relative expression level in transcriptome sequencing data and RT-qPCR. (B) Structure sketch for potential lncRNA-Gm2044 ORF verification. Tafa2-Flag and potential lncRNA-Gm2044 ORF1/ORF2/ORF3-Flag were inserted after the CMV/T7 promoter in the pcDNA3.1 (+) vector. (C) There was no protein band produced by the lncRNA-Gm2044 ORF1/ORF2/ORF3-Flag. Protein was isolated from 293T cells transfected by the indicated plasmid and then subjected to western blotting. Abbreviation: PC, positive control.

Liang et al., 2020). Furthermore, the abnormally high expression of lncRNA-Gm2044 was proven to be involved in nonobstructive azoospermia with spermatogonial arrest (Liang et al., 2019). At present, transgenic mice expressing lncRNA-Gm2044 in male germ cells using the *Stra8* promoter were constructed to explore the roles of upregulated lncRNA-Gm2044 in male fertility. The testicular/epididymal morphology and index, number of spermatozoa in the epididymis, and male fertility weren't affected in Gm2044-Tg mice. But, overexpression of lncRNA-Gm2044 in male germ cells partially impaired spermatogenesis. About one-fifth of the seminiferous tubules became small or twisted, and their spermatogenic cells were disordered and reduced in Gm2044-Tg mice.

The studies of differentially expressed genes of transcriptome sequencing have been widely used to explore the downstream signaling pathways and functions of lncRNA (Kurihara et al., 2017; Hong et al., 2021). The testes of lncRNA-NR\_038002 knockout mice were used to perform RNA-seq, and 82 differentially expressed genes were revealed, of which 69 were distributed on sex chromosomes (Hong et al., 2021). Overexpression of lncRNA-Tcam1 in spermatocyte GC-2 cells identified immune-related genes, *Tgtp2* and *Trim30a*, involved in spermatogenesis by RNA-seq (Kurihara et al., 2017). This study focused on the differentially expressed genes for the Gm2044-Tg and non-Tg using transcriptome sequencing, and 442 significantly upregulated genes and 147 significantly downregulated genes in Gm2044-Tg germ cells were identified. Furthermore, GO analysis showed that lncRNA-Gm2044 may regulate the processes of RNA\_metabolic\_process [GO:0016070], Central\_element [GO:0000801], Enzyme\_binding [GO:0019899], Protein\_binding [GO:0005515], Regulation\_of\_renal\_output\_by\_angiotensin [GO:0002019], Intracellular\_bridge [GO:0045171], and Enzyme\_regulator\_activity [GO:0030234]. In addition, KEGG analysis suggested that lncRNA-Gm2044 may involve the signaling pathways of RNA\_transport [mmu03040], Cell\_cycle [mmu04110], Renin-angiotensin\_system [mmu04614], and Chemokine\_signaling\_pathway [mmu04062] during spermatogenesis. In the future, the detailed molecular mechanisms of lncRNA-Gm2044 involved in these processes will be investigated.

Acrosome formation of sperm is critical for metamorphosis of spermatogenic cells during spermatogenesis, and abnormalities of acrosomal formation can lead to male infertility (Niu et al., 2016). Loss of ZPBPs in male mouse can damage the acrosome formation and sperm morphogenesis in spermatogenesis (Lin et al., 2007). The functional integrity of the plasma membrane of sperm is crucial to sperm motility and male fertility (Sabovic et al., 2020). The sperm plasma membrane protein ADAM family, ADAM2/3/4/6B, has been demonstrated to regulate sperm motility and egg-sperm binding by interacting with PRSS37 (Xiong et al., 2021). We analyzed differentially expressed gene sets, and the results showed that lncRNA-Gm2044 may participate in the processes of Acrosome\_assembly

[GO\_GO:0001675] and Sperm\_plasma\_membrane [GO\_GO:0097524]. Furthermore, the analysis revealed that *Zpbp*, *Nectin2*, *Tmf1* and *Sox30* possibly mediate the lncRNA-Gm2044 regulating Acrosome\_assembly. In addition, lncRNA-Gm2044 may be involved in the physiological function of Sperm\_plasma\_membrane by affecting *Adam2/4/6a/6b/21/24*, *Hsp90ab1*, *Ccr6*, and *Lzyl6* in male germ cells. The potential function of the above genes in lncRNA-Gm2044 overexpression partially impairing spermatogenesis need to be further studied.

Recent studies have demonstrated that many lncRNAs have the ability to encode protein peptides using their own ORFs. Then, the lncRNAs and/or peptides play important roles in disease occurrence and physiological processes (Choi et al., 2019; Xing et al., 2020). Also, some studies have proven that testicular-specific lncRNAs don't have the ability to encode protein peptides (Li et al., 2020). In this study, we predicted that three potential ORFs existed in the same strand of lncRNA-Gm2044, and these potential Gm2044 ORF1/ORF2/ORF3-Flag were inserted after the CMV/T7 promoter in the pcDNA3.1 (+) vector. The positive control (Tafa2-Flag) produced a clear protein band, but no protein band was produced by the Gm2044 ORF1/ORF2/ORF3-Flag, which demonstrated that the potential ORFs in lncRNA-Gm2044 do not have the ability to be translated into protein peptides and function as non-coding RNA.

In summary, transgenic mice expressing lncRNA-Gm2044 in male germ cells revealed that overexpression of lncRNA-Gm2044 partially impaired spermatogenesis and many signaling pathways involved in the regulation of spermatogenesis by lncRNA-Gm2044. lncRNA-Gm2044 works through functioning as a non-coding RNA, rather than encoding protein peptides. These findings suggest that the abnormally expressed lncRNAs in male germ cells may be a novel molecular target for the diagnosis and treatment of male infertility.

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Author contributions: Meng Liang and Xiaohua Liu designed the study and wrote the manuscript. Ke Hu, Yuanyuan Gao, Yifan Xu, Chaofan He and Kaixian Wang performed the experiments. Leina Li and Yaping Liao discussed the results.

## DISCLOSURES

The authors have no competing financial interests to declare.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at [doi:10.1016/j.psj.2022.101930](https://doi.org/10.1016/j.psj.2022.101930).

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