Long noncoding RNAs regulated spermatogenesis in varicocele-induced spermatogenic dysfunction

Shangren Wang | Jiaqi Kang | Yuxuan Song | Aiqiao Zhang | Yang Pan | Zhexin Zhang | Yuezheng Li | Shuai Niu | Li Liu | Xiaoqiang Liu

1Department of Urology, Tianjin Medical University General Hospital, Tianjin, China
2Department of Urology, Peking University People’s Hospital, Beijing, China
3Biomedical Pioneering Innovation Center (BIOPIC), School of Life Sciences, Peking University, Beijing, China
4Department of Neonatology, First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, Tianjin, China
5Department of Neonatology, National Clinical Research Center for Chinese Medicine Acupuncture and Moxibustion, Tianjin, China

Correspondence
Xiaoqiang Liu, Department of Urology, Tianjin Medical University General Hospital, 154 Anshan Rd, Heping District, Tianjin 300052, China.
Email: xiaoqiangliu1@163.com

Funding information
National Natural Science Funds of China, Grant/Award Number: 82171594; Zhao Yi-Cheng Medical Science Foundation, Grant/Award Number: ZYYFY2018031

Abstract
Objectives: To evaluate the expression, potential functions and mechanisms of long noncoding RNAs (lncRNAs) in the pathogenesis of varicocele (VC)-induced spermatogenic dysfunction.

Materials and Methods: We established a rat model with left experimental VC and divided rats into the sham group, the VC group, and the surgical treatment group (each group, n = 10). Haematoxylin and eosin (HE) staining and sperm quality were analysed to evaluate spermatogenesis function. LncRNA expression profiles were analysed using lncRNA-Seq (each group n = 3) and validated using quantitative real-time polymerase chain reaction (each group n = 10). Correlation analysis and gene target miRNA prediction were used to construct competing endogenous RNA network. The regulated signalling pathway and spermatogenic dysfunction of differentially expressed lncRNAs (DE lncRNAs) were validated by Western blot.

Results: HE detection and sperm quality analysis showed that VC could induce spermatogenic dysfunction. Eight lncRNAs were upregulated and three lncRNAs were downregulated in the VC group compared with the sham group and surgical treatment group. The lncRNA of NONRATG002949.2, NONRATG001060.2, NONRATG013271.2, NONRATG022879.2, NONRATG023747.2 were significantly negatively related to sperm quality, while NONRATG027523.1, NONRATG017183.2 and NONRATG023747.2 were positively related to sperm quality. The lncRNAs promote spermatogenic cell apoptosis and inhibit spermatogonia and spermatocyte proliferation and meiotic spermatocytes by regulating the PI3K–Akt signalling pathway.

Conclusion: DE lncRNAs may be potential biomarkers for predicting the risk of spermatogenic dysfunction in VC and the effect of surgical repair. These DE lncRNAs promote spermatogenic dysfunction by regulating the PI3K–Akt signalling pathway.

Abbreviations: Akt, protein kinase B; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma-2; PCNA, proliferating cell nuclear antigen; PI3K, phosphatidylinositol 3-kinase; PLZF, promyelocytic leukaemia zinc finger protein; REC8, recombination 8; STRA8, retinoic acid gene 8; SYCP3, synaptonemal complex protein 3.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2022 The Authors. Cell Proliferation published by John Wiley & Sons Ltd.
1 | INTRODUCTION

Varicocele (VC) is a common disease in male infertility in which the internal spermatic vein malformation is twisted, dilated, and elongated. The prevalence of VC in the general population is approximately 15%, and in infertile males, it is approximately 35%. VC is a common etiology of male infertility. Studies have shown that VC plays a role in decreased testicular function, leading to spermatogenic dysfunction and diminished testosterone levels. Some mechanisms may contribute to VC-induced spermatogenic dysfunction, including the ionic imbalance, high testicular temperature, neuroendocrine system dysfunction, hypoxia, chronic oxidative and disruption of the blood–testis barrier. Those chronic stress may reduce the function of spermatogenesis and damage the structure of spermatogenic cells such as DNA, RNA, lipids and proteins resulting in poor sperm quality. However, the exact mechanisms remain unclear and require more research.

Long noncoding RNAs (lncRNAs), a kind of noncoding RNA (ncRNA), are more than 200 nucleotides in length and lack functional protein-coding ability. Some studies have shown that lncRNAs play an essential role in the adjustment of gene expression and have broad functions in many critical biological processes, such as genomic imprinting, differentiation, apoptosis, nuclear organization, alternative splicing and nuclear import. LncRNAs participate in various disease processes, such as diabetes, cardiovascular disease and cancers. Wen et al. showed that testis-specific lncRNAs play an important role in late Drosophila spermatogenesis. Sanei-Ataabadi et al. found that oxidative-related lncRNAs are related to VC-connected male sterility. Although many lncRNAs exist in spermatogenesis, their expression and function in spermatogenic dysfunction induced by VC remain to be studied.

To understand the expression and function of lncRNAs in VC-induced spermatogenic dysfunction, in the present study, we performed RNA-seq to profile lncRNA expression in VC rats, and the results were validated for expression in the testis and relationships with sperm quality. The findings will contribute to understanding these mechanisms of spermatogenic dysfunction caused by VC and identifying new biomarkers for the diagnosis and treatment of VC-induced spermatogenic dysfunction.

2 | MATERIALS AND METHODS

2.1 | Animals

Thirty male Sprague Dawley rats aged 6–7 weeks were obtained from the Institute of Radiation Medicine, Chinese Academy of Medical Sciences. After 7 days of adaptive feeding, the rats were randomized into three groups: sham group (n = 10), VC group (n = 10), and surgical treatment group (n = 10). The research was supported by the Ethics Committee of Tianjin Medical University General Hospital (Approval No. IRB2021-DW-51).

Based on Turner’s previously published surgical protocol, we built a left experimental VC rat model. The sham group received a similar treatment without left renal vein obstruction. The VC rat models tested the sperm vein diameter at 8 weeks after modelling. Compared with the sham group, a more than twofold increase in the outer diameter of the left spermatic vein is considered a successful VC model. Meanwhile, the surgical treatment group received varicocelectomy at 8 weeks after modelling. The steps were as follows: open the abdomen layer by layer, separate the spermatic vein, ligate the vein with 4–0 silk thread, test the outer diameter of the left spermatic vein after ligating, and finally, close the abdomen layer by layer. Samples were obtained for next analysis at 4 weeks after varicocelectomy.

2.2 | Semen analysis

The left caudal epididymis was minced in phosphate-buffered saline, and the sperm were released after incubation for 5 min at 37°C. The sperm count and sperm motility, including progressive (PR) and non-progressive (NP) motility, were analysed using a computer-aided analysis system (Weil). Total motility is defined as the percentage of PR and NR sperm motility.

2.3 | Histological examination

Fresh testicular tissues were immobilized with 4% formalin for 1 day, dehydrated in the presence of increased ethanol concentrations, and embedded in paraffin for sectioning. The sections were stained with haematoxylin and eosin (HE) dye and observed under a light microscope.

2.4 | Total RNA extraction and lncRNA sequencing

We extracted total RNA of fresh testicular tissues with TRIzol (Invitrogen) and qualified it with an Agilent 2100 Bioanalyzer (Agilent Technologies), a NanoDrop spectrophotometer (Thermo Fisher Scientific) and 1% (wt/vol) agarose gels. Three rats from each group were randomly selected for lncRNA sequencing. High-throughput sequencing and subsequent data analysis were implemented by GENESKY Biotechnologies Inc. using the standard Illumina HiSeq 2500 platform.

2.5 | Identification of differentially expressed lncRNAs

The edgeR package was applied to identify the differentially expressed lncRNAs (DE lncRNAs) using the standard of log(fold change) > 1 and adjusted p < 0.05. We analysed the comparisons between the VC group versus the sham group and the VC group versus the surgical treatment group to generate DE lncRNAs. DE
IncRNAs were visualized by heatmaps and volcano maps drawn by the heatmap and ggplot2 packages. Overlapping dysregulated DE IncRNAs were screened for comparison between the two groups by Venny2.1 ([https://bioinfogp.cnb.csic.es/tools/venny/index.html](https://bioinfogp.cnb.csic.es/tools/venny/index.html)).

### 2.6 Construction of lncRNA–miRNA–mRNA network

We predicted interactions between lncRNAs and miRNAs by miRanda software with a perfect seed match. Full-length sequences of lncRNA and miRNA were selected. miRNA–mRNA interactions were identified by combining RNAhybird and miRanda software. Then, we screened pairs of positive correlations between the expression of lncRNAs and mRNAs, and the lncRNA–miRNA–mRNA competing endogenous RNA (ceRNA) network was obtained and visualized with Cytoscape v3.7.1 software. The cytoHubba plug-in was used to rank target genes and identify hub genes.

### 2.7 Protein–protein interaction network

We constructed protein–protein interaction (PPI) networks based on predicted target genes using the interactive gene retrieval tools (STRING) database[^19] and visualized them through Cytoscape v3.7.1.[^19]

### 2.8 Functional enrichment analysis

To investigate the biological function of DE IncRNAs, we performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses, including biological process, molecular function and cellular component analyses, for target genes and hub genes by Metascape.[^20] Terms with \( p < 0.05 \) were considered statistically significant.

### 2.9 Validation by quantitative real-time polymerase chain reaction

We extracted total RNA from testicular tissue samples using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Then, first-strand cDNA Synthesis Super Mix for qPCR (Yeasen) was used to amplify the cDNA. Subsequently, a SYBR Green PCR kit (Yeasen) was used for quantitative real-time polymerase chain reaction (RT-PCR) with a cDNA template on a real-time detector (MA-6000; Molarray). Quantifications were normalized to GAPDH and were analysed using the \( 2^{-\Delta \Delta C(t)} \) method. Table 1 shows the primer sequences.

### Table 1: Primer pairs used in this study

| IncRNAs          | Primer sequence (5’–3’) |
|------------------|-------------------------|
| NONRATG001060    | Forward: TCCAGTTAACGACCTCTCTC  
                 | Reverse: GTCTGCTAGCTCTTGAGCAAA |
| NONRATG002949    | Forward: AGAACCGCACTAGTACGAGA  
                 | Reverse: TCCAGTTAACGACCTCTCTC  |
| NONRATG005667    | Forward: ATGACAGCGTGTTGACAGTGT  
                 | Reverse: CATTCTGGTCTGACAGTACGACAA |
| NONRATG007482    | Forward: TCCTGCTGGATCAACGAGAAGA  
                 | Reverse: ACTCTGCTGGATCAACGAGAAGA  |
| NONRATG010686    | Forward: CATTACCTGGCCTTCGATTAGC  
                 | Reverse: GCAAGCACAAGAATTCGATCCA  |
| NONRATG013271    | Forward: TGGCTCAGAAGAGATACGAGA  
                 | Reverse: CAGGAGATCGCAGCAATCCAG  |
| NONRATG017183    | Forward: TCTTCTCTCTCTCTGCTCTCT  
                 | Reverse: ACACTCCATTACCCCACACTGAGAT |
| NONRATG028279    | Forward: TTCTGCTGGATCAACGAGAAGA  
                 | Reverse: ACTCAGGAAGACTGCTACTGGAAGAT |
| NONRATG023424    | Forward: CTGTCTTCTGATCGTTCTACCC  
                 | Reverse: CAAATTCGCTGCCATCCCTTCC |
| NONRATG023747    | Forward: ATCTCTCAACAGTGGTCTCTA  
                 | Reverse: GACTCTGATCTGCCCTGGCTTAC |
| NONRATG027523    | Forward: AGGAGGCTGATTACGACATGG  
                 | Reverse: AGTGGCTGATTACGAGGAGGAAG |
| GAPDH            | Forward: GGCAAGTTCAGGCGACAGC  
                 | Reverse: CAGGCCAGTACGCCAGCAC |
2.10 Apoptosis tests

Terminal deoxynucleotidyl transferase nick end labeling (TUNEL) staining was performed using the TUNEL Assay Kit (KGA7073; 7Sea Boitech) according to the manufacturer's instructions. Pictures were taken under a microscope for green fluorescence generated by TUNEL-positive cells and red fluorescence generated by total DNA. TUNEL positivity was calculated by dividing the total number of TUNEL-positive cells by the number of nuclei.
### TABLE 2  Basic parameters and semen analysis in rats

| Group                      | Sham group      | VC group         | Surgical treatment group | p Value<sup>a</sup> | p Value<sup>b</sup> |
|---------------------------|-----------------|------------------|--------------------------|---------------------|---------------------|
| Body weight at 8 weeks (g)| 361.10 ± 2.09   | 362.90 ± 1.43    | 361.70 ± 1.47            | 0.4866              | 0.5660              |
| Lift testicular weight (g)| 1.79 ± 0.04     | 1.63 ± 0.02      | 1.68 ± 0.02              | 0.0036              | 0.1372              |
| Right testicular weight (g)| 1.75 ± 0.04   | 1.65 ± 0.03      | 1.67 ± 0.03              | 0.0602              | 0.5643              |
| Left sperm vein diameter (mm)| 0.372 ± 0.01 | 1.597 ± 0.02     | 0.602 ± 0.02             | <0.0001             | <0.0001             |
| Sperm concentration (×10^6) | 234.4 ± 9.58   | 201.2 ± 8.39     | 222.8 ± 6.90             | 0.0178              | 0.0623              |
| Progressive motility (PR%) | 44.91 ± 3.10   | 33.39 ± 1.96     | 42.30 ± 1.911            | 0.0057              | 0.0044              |
| Total motility (PR + NP%)  | 54.83 ± 3.10   | 43.76 ± 2.36     | 50.29 ± 2.07             | 0.0108              | 0.0519              |

Abbreviations: NP, nonprogressive; PR, progressive; VC, varicocele.

<sup>a</sup>p Value for Sham group vs. VC group.

<sup>b</sup>p Value for VC group vs. surgical treatment group.

---

**(A)** Sham  VC  Surgical treatment

**(B)**

*HE staining of rat testicular tissues, basic parameters and semen analysis in rats. (A) Representative HE staining image of rat testicular tissues (×200). (B) Testicular weight of lift, (C) sperm count, (D) sperm total motility, and (D) PR% of sperm. *p <0.05; **p <0.01; HE, haematoxylin and eosin; NS, not significant; PR, sperm progressive motility; surgical treatment, surgical treatment group; sham: sham group; VC, varicocele group.*
FIGURE 3  The expression profiles of lncRNAs in VC rats. (A) Heatmap for sham group versus VC group. (B) Heatmap for surgical treatment group versus VC group. (C) Volcano map for VC group versus sham group. (D) Volcano map for VC group versus surgical treatment group. (E) Venn diagram showing upregulation of eight DE lncRNAs among the surgical treatment group versus VC group and VC group versus sham group comparisons. (F) Venn diagram showing downregulation of three DE lncRNAs among the surgical treatment group vs. VC group and VC group vs. sham group comparisons comparisons. Down, downregulation; NOT, not significant; Sham, sham group; Surgical treatment, surgical treatment group; Up, upregulation; VC, varicocele group
Western blot

Testicular tissues of rats were prepared using radioimmunoprecipitation assay buffer containing protease inhibitor. BCA (Solarbio) was used to test protein concentrations. The protein was separated by electrophoresis and transferred to membranes. The following primary antibodies were used for incubation with membranes: PI3K (1:1000; Abcam), Akt (1:1000; Affinity Biosciences), p-Akt (1:1000; Affinity Biosciences), caspase-9 (1:1000; Affinity Biosciences), Bcl-2 (1:1000; Affinity Biosciences), Bax (1:1000; Affinity Biosciences), PCNA

### Table 3: Key lncRNAs in the differential expression analysis

| lncRNAs             | VC group vs. Sham group | VC group vs. Surgical treatment group |
|---------------------|-------------------------|--------------------------------------|
|                     | logFC | Adjusted p value | logFC | Adjusted p value |
| NONRATG007482.2     | 1.332 | 0.012            | 1.165 | 0.050            |
| NONRATG002949.2     | 1.578 | 0.014            | 2.317 | 0.001            |
| NONRATG001060.2     | 9.918 | 0.001            | 9.752 | 0.004            |
| NONRATG013271.2     | 1.201 | 0.018            | 1.234 | 0.041            |
| NONRATG022879.2     | 1.293 | 0.003            | 1.160 | 0.029            |
| NONRATG023424.2     | 1.650 | 0.003            | 1.798 | 0.003            |
| NONRATG005667.2     | 1.347 | 0.024            | 1.450 | 0.021            |
| NONRATG010686.2     | 1.363 | 0.028            | 1.484 | 0.048            |
| NONRATG027523.1     | −9.253| <0.001           | −8.369| 0.004            |
| NONRATG017183.2     | −7.654| 0.004            | −8.541| <0.001           |
| NONRATG023747.2     | −1.505| 0.010            | −2.128| <0.001           |

Abbreviation: VC, varicocele.

### Figure 4: The regulatory lncRNA–miRNA–mRNA network. Red: lncRNAs, purple: miRNAs, cyan-blue: targeted genes
(1:1000; Affinity Biosciences), PLZF (1:1000; Affinity Biosciences), REC8 (1:1000; Bios), STRA8 (1:1000; Affinity Biosciences), SYCP3 (1:1000; Affinity Biosciences), GAPDH (1:1000). Then, the membranes were incubated with a secondary antibody (Bioss). Subsequent visualization with a chemiluminescent imaging system was performed (KPL).

### 3. RESULTS

The research flow chart is summarized in Figure 1. As showed in Table 2, 8 weeks after modelling, VC model rats showed significant dilation of the spermatic vein and body weights among the three groups were not significantly different. The weight of the left testis in the sham group was higher than that in the VC group ($p < 0.01$, Figure 2B) and was not significantly different between

![Functional enrichment analysis of target genes](image)

**TABLE 4** Functional enrichment analysis of target genes

| Category                  | Term                     | Description                              | FDR      | lnTerm_InList |
|---------------------------|--------------------------|------------------------------------------|----------|---------------|
| GO biological processes   | GO:0002252               | Immune effector process                  | 1.38E-11 | 53/764        |
| GO biological processes   | GO:0001816               | Cytokine production                      | 2.95E-08 | 46/737        |
| GO biological processes   | GO:0045087               | Innate immune response                   | 8.08E-07 | 40/659        |
| GO biological processes   | GO:0002697               | Regulation of immune effector process    | 1.74E-06 | 31/434        |
| GO biological processes   | GO:0031347               | Regulation of signal transduction        | 1.78E-06 | 36/571        |
| GO biological processes   | GO:0051607               | Defence response to virus                | 1.45E-05 | 21/236        |
| GO biological processes   | GO:0099991               | Response to extracellular stimulus       | 2.99E-05 | 39/746        |
| GO biological processes   | GO:0050900               | Leukocyte migration                      | 3.66E-05 | 26/378        |
| GO biological processes   | GO:0050865               | Regulation of cell activation            | 6.60E-05 | 34/619        |
| KEGG pathway              | ko04060                  | Cytokine–cytokine receptor interaction   | 8.64E-05 | 19/226        |
| GO biological processes   | GO:0051384               | Response to glucocorticoid               | 8.81E-05 | 22/300        |
| GO biological processes   | GO:0044057               | Regulation of system process             | 2.96E-04 | 33/647        |
| GO biological processes   | GO:0071887               | Leukocyte apoptotic process              | 3.43E-04 | 14/140        |
| GO biological processes   | GO:0035150               | Regulation of tube size                  | 5.46E-04 | 15/168        |
| GO biological processes   | GO:0048771               | Tissue remodeling                        | 5.53E-04 | 17/215        |
| GO biological processes   | GO:0071346               | Cellular response to interferon-gamma    | 5.53E-04 | 13/127        |
| GO biological processes   | GO:0043408               | Regulation of MAPK cascade               | 9.64E-04 | 34/729        |
| GO biological processes   | GO:006979                | Response to oxidative stress             | 9.75E-04 | 28/538        |
| KEGG pathway              | rno05200                 | Pathways in cancer                       | 9.80E-04 | 23/390        |
| GO biological processes   | GO:0001906               | Cell killing                             | 9.80E-04 | 17/229        |

Data were represented by the mean ± SD. Unpaired Student’s t-test was used to compare parameter data between groups. The Mann–Whitney rank-sum test was used for nonparametric data. We carried out a Pearson correlation analysis between DE lncRNA expression and semen parameters. We carried out all statistical analyses using GraphPad Prism 8.0 (GraphPad Software). A result of $p < 0.05$ was regarded with statistical significance.

### 2.12 Statistical analysis

The research flow chart is summarized in Figure 1. As showed in Table 2, 8 weeks after modelling, VC model rats showed significant dilation of the spermatic vein and body weights among the three groups were not significantly different. The weight of the left testis in the sham group was higher than that in the VC group ($p < 0.01$, Figure 2B) and was not significantly different between
The sperm count, total motility and PR motility of rats in the sham group were apparently higher than those in the VC group (Figure 2C, D). The sperm count and total motility in rats after surgical treatment did not increase significantly (Figure 2C, D), but the number of PR motility sperm increased significantly compared with the VC group (p < 0.01, Figure 2E).

### 3.1 | HE staining of rat testicular tissues

As shown in Figure 2A, HE staining analysis further reflected that the counts of spermatogonia, spermatocytes and round spermatids in the seminiferous tubules of the VC group were significantly reduced, while those in the surgical treatment group were significantly increased.

### 3.2 | Identification of the coexpression of DE IncRNAs in the VC group

To elucidate the mechanism of impaired sperm quality in the testes of VC rats in more detail, entire testis samples for IncRNA sequencing were collected. Compared with those in the sham group, 244 upregulated IncRNAs and 27 downregulated DE IncRNAs were detected in the VC group (Figure 3A, C). In the comparison between the VC group and the surgical treatment group, we identified 82 DE IncRNAs, including 42 upregulated

---

### Table 5 | Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway functional enrichment analysis

| Term                           | Description                                                                 | p Value | Symbols |
|--------------------------------|-----------------------------------------------------------------------------|---------|---------|
| rno05200 | Pathways in cancer | 2.82E−06 | Hgf, Cxcl12, Fgrf2, Mitf, Stat1, Pdgfa, Pdgfra, Casp3, F2r, Rock2, Egln3, Fzd1, Kitlg, Gng11, Tgf8, Rorg, Gng10, Wnt12b, Wnt11, Fas, Wnt9b, Csf1r, Fos, Tlr4, Pxn, Mapk11, Wls, Osr1, Hnf1a, Jak3, Meis1, Edn1, Tnrsf11b, Ir3, Rbp4, Hoxd4, Adm |
| rno05162 | Measles | 6.58E−05 | Stat1, Jak3, Tlr4, E2f2ak2, Oas1a, Fas, Mx2, Fcr2b, Irf7, Ddx58, Ifih1 |
| rno05164 | Influenza A | 9.47E−04 | Stat1, Tlr4, E2f2ak2, Radd2, Oas1a, Fas, Mx2, Irf7, Ddx58, Il33, Ifih1, Mapk11 |
| rno05161 | Hepatitis B | 3.45E−04 | Stat1, Casp3, Tlr4, Tgfb2, Fas, Irf7, Ddx58, Fos, Ifih1, Crib5 |
| rno05168 | Herpes simplex infection | 2.80E−03 | RT1-M3-1, Tap1, Stat1, Casp3, E2f2ak2, Oas1a, Fas, Irf7, Ddx58, Fos, Ifih1 |
| rno04060 | Cytokine–cytokine receptor interaction | 1.07E−07 | Hgf, Il6r, Il9r, Cxcl12, Pdgfa, Pdgfra, Tnrsf11b, Csf3, Ccl11, Kitlg, Tgf8, C3cd1, Ccr5, Fas, I13ra1, Ccl21, I18r1, Csf1r, I17r |
| rno04640 | Haematopoietic cell lineage | 7.99E−03 | Il6r, Il9r, Mme, Csf3, Kitlg, Csf1r |
| rno04151 | PI3K–Akt signalling pathway | 2.78E−05 | Hgf, Il6r, Fgrf2, Pdgfa, Pdgfra, Jak3, Spp1, F2r, Ir3, Csf3, Itgb7, Tlr4, Kitlg, Gng11, Prkkaa2, Gng10, Csf1r, Ephb2, Crib5 |
| rno04014 | Ras signalling pathway | 5.39E−03 | Hgf, Fgrf2, Pld1, Pdgfa, Pdgfra, Plag2, Kitlg, Gng11, Gng10, Csf1r, Ephb2 |
| rno05205 | Proteoglycans in cancer | 6.75E−04 | Hgf, Casp3, Rock2, Tlr4, Fzd1, Tgfb2, Wnt2b, Wnt11, Fas, Wnt9b, Pxn, Mapk11 |
| rno04550 | Signalling pathways regulating the pluripotency of stem cells | 1.51E−03 | Hnt1a, Fgrf2, Jak3, Fzd1, Wnt2b, Wnt11, Wnt9b, Meis1, Mapk11 |
| rno04916 | Melanogenesis | 3.11E−03 | Edn1, Mitf, Fzd1, Kitlg, Wnt2b, Wnt11, Wnt9b |
and 40 downregulated DE lncRNAs (Figure 3B,D). Venn diagram analysis showed that 11 DE lncRNAs overlapped between the two comparisons (Figure 3E,F). Specifically, eight DE lncRNAs were upregulated and three DE lncRNAs were downregulated in the VC group (Table 3).

### 3.3 The regulatory lncRNA–miRNA–mRNA network

LncRNAs can act as miRNA sponges via ceRNA networks to regulate miRNA-targeted gene expression. Mo-miR-301a-5p and
mo-miR-328a-5p, which can potentially bind to four DE lncRNAs, were identified and are shown in Figure 4. The predicted mRNAs were selected as potential target genes in at least two of the databases used (RNAhybrid and miRanda). Finally, lncRNA–miRNA–mRNA regulatory networks were constructed based on 4 lncRNAs, 2 miRNAs, and 12 mRNAs (Figure 4).

### 3.4 Functional enrichment analysis of target genes

For further research, the biological processes and pathways based on the established ceRNA network were explored. The DE lncRNAs were closely related to inflammation or immune-associated biological processes, apoptosis and oxidative stress, such as immune effector processes, cytokine production, innate immune response, defensive reaction regulation, leukocyte apoptotic process and response to oxidative stress (Figure 5 and Table 4). Similarly, several KEGG pathways were identified, including cytokine–cytokine receptor interactions, pathways in cancer and the PI3K–Akt signalling pathway (Figure 6 and Table 5). Overall, these results suggest that the DE lncRNAs are correlated with the behaviour of VC.

### 3.5 Construction of the PPI network and identification of hub genes

As shown in Figure 7, we predicted a PPI network to display the interactions of target genes through the STRING database. Then, we recognized the top 10 genes with the highest association in the PPI network by the cytoHubba plug-in (Figure 8A and Table 6). Functional enrichment analysis by Metascape indicated that eight GO terms, including reaction to lipopolysaccharide (GO:0032496), nitric oxide synthase biosynthetic process (GO:0051767), control of the response to cytokine stimulus (GO:0060759), cytokine-mediated signalling pathway (GO:0019221), neuron death (GO:0070997), positive control of cytokine production (GO:0001819), interferon-alpha production (GO:0032727) and response to external stimulus (GO:0032103), and two pathways, including toxoplasmosis (rno05145) and innate immune system (R-RNO-168249), were significantly related to hub genes (Figure 8B and Table 7).
Validation of DE lncRNAs by RT-PCR. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. DE lncRNAs, differentially expressed lncRNAs; NS, not significant; RT-PCR, quantitative real-time polymerase chain reaction; Sham, sham group; Surgical treatment, surgical treatment group; VC, varicocele group.
Validation of DE lncRNAs by RT-PCR

Furthermore, we verified the expression of DE lncRNAs in testicular tissues of VC rats by RT-PCR. As illustrated in Figure 9, the relative expression of NONRATG002949.2, NONRATG001060.2, NONRATG013271.2, NONRATG022879.2, NONRATG023424.2, NONRATG005667.2 and NONRATG010686.2 was higher in the VC group than in the other two groups (p < 0.05). The relative expression of NONRATG027523.1, NONRATG017183.2 and NONRATG023747.2 in the VC group was downregulated significantly (p < 0.05). No difference in NONRATG007482.2 expression was found (p > 0.05).
To illustrate the effect of DE lncRNAs on the semen quality of VC rats, we performed a correlation analysis between the DE lncRNA expression and sperm count and motility. As shown in Figures 10 and 11, the relative expression of NONRATG002949.2, NONRATG001060.2, NONRATG013271.2, NONRATG022879.2, NONRATG023424.2, NONRATG005667.2 and NONRATG010686.2 was significantly negatively related to sperm count and the percentage of PR, while NONRATG027523.1, NONRATG017183.2 and NONRATG023747.2 showed the opposite trend \( (p < 0.05) \).

**FIGURE 11** Correlation between DE lncRNA expression and the percentage of PR. DE lncRNAs, differentially expressed lncRNAs; PR, sperm progressive motility

### 3.7 Correlation between DE IncRNA expression and sperm quality

To illustrate the effect of DE IncRNAs on the semen quality of VC rats, we performed a correlation analysis between the DE IncRNA expression and sperm count and motility. As shown in Figures 10 and 11, the relative expression of NONRATG002949.2, NONRATG001060.2, NONRATG013271.2, NONRATG022879.2, NONRATG023424.2, NONRATG005667.2 and NONRATG010686.2 was significantly negatively related to sperm count and the percentage of PR, while NONRATG027523.1, NONRATG017183.2 and NONRATG023747.2 showed the opposite trend \( (p < 0.05) \).
No apparent association was exhibited between NONRATG007482.2 expression and sperm quality (p > 0.05).

3.8 Validation of regulated spermatogenic cell apoptosis by DE lncRNAs by TUNEL staining of rat testicular tissues

We performed TUNEL staining of rat testis tissue to investigate the regulation of DE lncRNAs on spermatogenic cell apoptosis. The TUNEL assay results showed that the percentage of apoptotic cells in the testicular tissue of VC rats increased but decreased after surgical intervention (Figure 12A,B). The DE lncRNAs regulate spermatogenic cell apoptosis in VC, and surgical treatment significantly improves apoptosis.

3.9 Validation of regulated signalling pathways, spermatogenic cell apoptosis and proliferation, and meiotic spermatocytes of DE lncRNAs by Western blot

To validate the downstream signalling pathways and phenotypes, according to the DE lncRNAs functional enrichment and KEGG analyses (Figure 6), we selected the PI3k–Akt signalling pathway to validate by Western blot. More and more studies have shown that the
PI3k–Akt signalling pathway regulated cell apoptosis, proliferation and spermatocytes in spermatogenesis. According to the DE lncRNA functional enrichment, TUNEL, and HE staining results, we selected the phenotypes of spermatogenic cell apoptosis, spermatogenic cell proliferation and meiotic spermatocytes to validate by Western blot. The results are shown in Figure 13. We found that the expression of PI3K did not change in the three groups \((p > 0.05)\), and the expression of Akt and p-Akt was decreased in the VC group compared with the sham group \((p < 0.05)\) and partly restored in the surgical treatment group \((p > 0.05)\). The expression of caspase-9 and Bax was increased and Bcl-2 was decreased in the VC group compared with the sham group and restored after surgical treatment \((p < 0.05)\). The expression of PCNA and PLZF was decreased in the VC group compared with the sham group and restored after surgical treatment \((p < 0.05)\).
The expression of REC8 and STRA8 was decreased in the VC group compared with the sham group. STRA8 expression was restored after surgical treatment \((p < 0.05)\), but REC8 showed no significant changes after surgical treatment \((p > 0.05)\). The expression of SYCP3 did not change in the three groups \((p > 0.05)\). These results suggested that DE lncRNAs promote spermatogenic cell apoptosis and inhibit spermatogonia and spermatocyte proliferation and meiotic spermatocytes by regulating the PI3K–Akt signalling pathway (Figure 14).

### 3.10 Identification of the coexpression of DE lncRNAs in the sham group

Compared with lncRNAs in the sham group, 244 upregulated lncRNAs and 27 downregulated DE lncRNAs were detected in the VC group (Figure S1A,C). In the comparison between the sham group and the surgical treatment group, we identified 51 DE lncRNAs, including 42 upregulated and 9 downregulated DE lncRNAs (Figure S1B,D). Venn diagram analysis showed that 19 DE lncRNAs overlapped between the two comparisons (Figure S1E,F). Specifically, all the 19 DE lncRNAs were upregulated and no DE lncRNAs were downregulated in the sham group.

### 4 DISCUSSION

VC is a common risk factor for male infertility.\(^1\) However, some people with VC have infertility or negative sperm quality, whereas others do not. The connection between VC and spermatogenic dysfunction is still controversial. lncRNAs play various functions in many diseases through signalling pathways.\(^22,23\) Previous studies have shown that lncRNAs play roles in VC-related male infertility through the regulation of hypoxia responses.\(^23\) Although a large number of lncRNAs have been found in the testis, their biological function remains to be
further studied in various diseases. There are few studies on lncRNA expression patterns in VC-induced spermatogenic dysfunction. Thus, how lncRNAs are expressed during spermatogenesis before and after VC surgical repair must be further understood.

In the present study, lncRNA sequencing technologies were used to analyse lncRNA expression in rats with VC. Our results showed that lncRNA expression was different in the VC group compared with the sham group or surgical treatment group. We considered the coexpression DE lncRNAs in VC group would be the key lncRNAs in VC-induced spermatogenic dysfunction. So, we analysed and validated the functions of the eight lncRNAs were significantly coincreased and three lncRNAs were significantly codecreased in the VC group versus sham group and surgical treatment group.

We validated the expression of the 11 lncRNAs in the three groups using RT-PCR. The results were in accord with the RNA-seq data analysis, except for lncRNA NONRATG007482.2. Then, we constructed a ceRNA network and suggested that 4 lncRNAs potentially interact with 2 miRNAs (miR-301a-5p and miR-328a-5p) and 12 mRNAs, and we conducted functional enrichment analysis of target genes and KEGG analyses. These biological processes are primarily involved in inflammation, oxidative stress and cell apoptosis, and so on. Depending on functional enrichment and KEGG analyses, we selected PI3K–Akt signalling pathway and the phenotypes of spermatogenic cell apoptosis, spermatogenic cell proliferation and meiotic spermatocytes to validate by Western blot. And the results showed that DE lncRNAs promoted spermatogenic cell apoptosis, and inhibit spermatogenic cell proliferation and meiotic spermatocytes by regulating the PI3K–Akt signalling pathway and other mechanisms.

LncRNAs are important mediators of the ceRNA regulatory network, and they can absorb miRNAs and regulate the expression of target genes. We constructed a ceRNA network and suggested that 4 lncRNAs potentially interact with 2 miRNAs (miR-301a-5p and miR-328a-5p) and 12 mRNAs. We identified more VC-induced spermatogenic dysfunction and surgical repair-specific ceRNA pairs than previous studies. In previous studies, miRNAs as biomarkers for VC have been widely discussed. Xu et al. analysed the expression of miR-210-3p in patients with VC and found that the level of miR-210-3p in seminal plasma of patients with VC was 2.18 times higher than that of healthy people. Zhi et al. found that miR-192a could be a predictive factor for the spermatogenic status of patients after VC repair. However, studies of the functions of miR-301a-5p and miR-328a-5p in VC or spermatogenesis are still lacking. Our study may provide two potential diagnostic and therapeutic candidates for VC-induced spermatogenic dysfunction. miR-301a-5p and miR-328a-5p participate in biological processes in many other diseases. Wang et al. found that lncRNA EPB41L4A-AS2 sponges miR-301a-5p and inhibits hepatocellular carcinoma development. The expression of miR-301a-5p was certified in gastric cancer tissues, and high miR-301-5p expression was found to be associated with the aggressiveness of gastric cancer. Huo et al. revealed that downregulated lncRNA-MIAT could activate miR-328a-5p against erectile dysfunction in diabetes mellitus rats. Through animal experiments, miR-328a-5p was found to be downregulated in a rat model of acute kidney injury induced by contrast agents.

Through functional enrichment analysis of target genes and KEGG analyses, we identified several signalling pathways and biological processes related to these lncRNAs. Our results suggested that DE lncRNAs were predominantly enriched in ‘immune effector process,’ ‘regulation of immune effector process,’ ‘leucocyte apoptotic process,’ and ‘response to oxidative stress.’ Most DE lncRNAs participated in ‘pathways in cancer,’ ‘PI3K–Akt signalling pathway’ and ‘Ras signalling pathway.’ These biological processes are primarily involved in inflammation, oxidative stress and cell apoptosis.

Many studies have shown that inflammatory mechanisms play important roles in VC. IL-1α and IL-1β have been reported to be increased in the VC model. Camargo et al. found that IL-1, IL-18 and caspase-1 decreased in semen after varicocelectomy by ELISAs. Micheli et al. tested seminal plasma samples and found that sperm apoptosis, IL-6, and TNF-α were increased in VC patients. Zeinali et al. measured IL expression in 75 infertile men with VC and showed that IL-18 increased and activated neutrophils and oxygen species in infertile patients with VC. These available studies and our present study data support that inflammation may play an essential role in the progression of spermatogenic dysfunction in VC.

We also found that lncRNAs and miRNAs are involved in the adjustment of oxidative stress, which plays an important role in VC-associated spermatogenic dysfunction. The relationship between oxidative stress and sperm damage in VC patients has been investigated by Ammar et al., who found that impaired seminal antioxidant capacity and elevated seminal levels of lipid peroxidation may contribute to the aetiology of nuclear sperm DNA damage in VC patients. Ata-Abadi et al. used RNA sequencing datasets from Gene Expression Monibus to identify hypoxia-responding lncRNAs, evaluated the expression of lncRNAs by RT-PCR and analysed their expression in patients with VC. The results showed that these lncRNAs, including MIR210HG and MLLT4-AS1, were positively correlated with oxidative stress and negatively correlated with sperm quality in men with VC. Oxidative stress-related expression patterns of miR-21, miR-34a and miR-122a were found to be decreased among patients with severe VC, particularly those with defective spermatogenesis in Ashrafzade et al.’s study.

The DE lncRNAs in our present study were not reported in a previous study of VC. These lncRNAs may be potential novel biomarkers for predicting the risk of spermatogenic dysfunction in VC and the effect of surgical repair. However, we randomly selected three rats from each group for lncRNA sequencing, the sample size for RNA-seq was small. The sample-to-sample variability may affect the stability of our study. Therefore, we further validated the expression of DE lncRNAs by RT-PCR and evaluated the correlation of the expression of the key lncRNAs with sperm quality. We found that a total of seven lncRNAs were negatively correlated with total sperm count and sperm PR motility, while three lncRNAs were positively correlated. The lncRNAs as biomarkers for predicting the risk of spermatogenic dysfunction in VC have been reported in previous studies. A study indicated that lncRNAs SLC-AS6 and SLC-AS7 were negatively correlated with sperm count and motility in male infertile sperm samples associated with VC. Zhao et al. found that lncRNA gadd7 could
promote the apoptosis of mouse spermatocytes in mice with VC-induced infertility.\textsuperscript{15}

To validate the downstream signalling pathways and phenotypes, according to the KEGG analyses, we selected the PI3K–Akt signalling pathway to validate by Western blot. We found that the expression of p-Akt protein decreased in the testes of VC rats. Previous studies showed the same results as our study of PI3K–Akt signalling pathway regulated spermatogenesis.\textsuperscript{40–42} Aquila et al. study found that estradiol could enhance phosphorylation of the protein Akt, which was regarded as a germ cell survival factor in the human testis.\textsuperscript{43} Dube et al. tested the epididymis tissues by RT-PCR, and found that decreased expression of epidermal growth factor increasing PI3K–Akt signalling pathway regulated the specific luminal microenvironment necessary for the creation of fertilizing-competent spermatozoa.\textsuperscript{44} Wang et al.\textsuperscript{45} used a VC rat model study and found that the PI3K–Akt signalling pathway plays a regulatory role in VC-induced spermatogenesis disorder. Zhao et al.\textsuperscript{40} performed in vitro experiments and found that the PI3K–Akt signalling pathway participates in regulating spermatogonial cell apoptosis and proliferation. A previous study indicated that lncRNAs also induce spermatogenic cell apoptosis in VC patients.

According to DE lncRNA functional enrichment, TUNEL and HE staining results, we selected the phenotypes of spermatogenic cell apoptosis, spermatogenic cell proliferation and meiotic spermatocytes to validate by Western blot. We found that the expression of the apoptosis-promoting proteins caspase-9 and Bax was increased, and the apoptosis-inhibiting protein Bcl-2 was decreased in the testes of VC rats. The TUNEL assay results indicated that the percentage of apoptotic spermatogenic cells in the testis tissue of VC rats increased and decreased after surgical intervention. Some apoptotic mechanisms were believed to be connected with VC, originating in the mitochondria of spermatocytes and working in the nucleus in Wu et al.’s study.\textsuperscript{9} Zhao et al. found that lncRNA gadd7 was upregulated in the semen of VC patients, and in an in vitro study indicated that over-expression of lncRNA gadd7 induced the apoptosis of spermatocytes and suppressed GC-1 and GC-2 cell proliferation.\textsuperscript{46,47}

We detected the expression of PCNA and PLZF, two biomarkers associated with spermatogonia and spermatocyte proliferation and differentiation.\textsuperscript{48,49} We found that the expression of PCNA and PLZF was decreased in VC rats and restored after surgical repair. STRA8, REC8 and SYCP3 are biomarkers associated with meiotic spermatocytes.\textsuperscript{49,50} Our results showed that the expression of STRA8 and REC8 was decreased in VC rats. STRA8 is a biomarker for entry of germ cells into meiotic prophase I, and REC8 is a meiotic marker gene.\textsuperscript{49,50} These results indicated that DE lncRNAs promote spermatogenic cell apoptosis and inhibit spermatogonia and spermatocyte proliferation and meiotic spermatocytes via the PI3K–Akt signalling pathway, which may affect subsequent spermatogenesis processes and sperm quality in VC.

Our study provides a foundation for the expression signature of lncRNAs at different stages of VC require more systemic investigations. Therefore, more studies based on larger sample sizes and in different stages of VC are necessary in the future.

5 CONCLUSION

Our study provides a foundation for the expression signature of lncRNAs to understand the molecular mechanisms in VC-induced spermatogenic dysfunction and surgical repair. Ten DE lncRNAs were associated with sperm quality in VC. The 10 DE lncRNAs may be potential novel biomarkers for predicting the risk of spermatogenic dysfunction in VC and the effect of surgical repair. These DE lncRNAs promote spermatogenic cell apoptosis and inhibit spermatogonia and spermatocyte proliferation and meiotic spermatocytes by regulating the PI3K–Akt signalling pathway. The functions of lncRNAs in VC-induced spermatogenic dysfunction require more systemic investigations in the future.

ACKNOWLEDGEMENTS

This study was funded by National Natural Science Funds of China (82171594) and Zhao Yi-Cheng Medical Science Foundation (ZYYFY2018031).

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Xiaoqiang Liu conceived the project. Shangren Wang, Jiaqi Kang, Yuxuan Song, Aiqiao Zhang and Yang Pan performed the experiment and wrote the manuscript. Zhixin Zhang and Yuezheng Li analysed data. Li Liu, Shuai Niu and Xiaoqiang Liu provided the resources. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Xiaoqiang Liu https://orcid.org/0000-0003-3524-6783

REFERENCES

1. Kang C, Punjani N, Lee RK, Li PS, Goldstein M. Effect of varicoceles on spermatogenesis. Semin Cell Dev Biol. 2022;121:114–124.
2. Gill K, Kups M, Harasny P, et al. The negative impact of varicocele on basic semen parameters, sperm nuclear DNA dispersion and oxidation-reduction potential in semen. Int J Environ Res Public Health. 2021;18(11):5977.
3. Agarwal A, Majzoub A, Parekh N, Henkel R. A schematic overview of the current status of male infertility practice. World J Mens Health. 2020;38(3):308–322.
Zhang L, Zhao X, Wang W. lncRNA and mRNA sequencing of the left testis in experimental varicocele rats treated with Morinda officinalis polysaccharide. Exp Ther Med. 2021;22(4):1136.

Xu Y, Zhang Y, Yang Y, Liu X, Chen X. Seminal plasma miR-210-3p is a biomarker for screening dyszoospermia caused by varicocele. Andrologia. 2019;51(5):e13244.

Zhang E, Liang GQ, Li P, et al. Seminal plasma miR-192a: a biomarker predicting successful resolution of nonobstructive azoospermia following varicocele repair. Asian J Androl. 2018;20(4):396-399.

Wang YG, Wang T, Shi M, Zhai B. Long noncoding RNA EPB41L4A-AS2 inhibits hepatocellular carcinoma development by sponging miR-301a-5p and targeting FOXL1. J Exp Clin Cancer Res. 2019;38(1):153.

Huang Y, Du X, Chen X, et al. MiR-301a-5p/SCN1 promotes gastric cancer progression via regulating STAT3 and NF-κB signaling. J Cancer. 2021;12(18):5394-5403.

Huo W, Hou Y, Li Y, Li H. Downregulated IncRNA-MIAT confers protection against erectile dysfunction by downregulating lipoprotein lipase via activation of miR-328a-5p in diabetic rats. Biochim Biophys Acta Mol Basis Dis. 2019;1865(6):1226-1240.

Liu Y, Liu B, Liu Y, et al. MicroRNA expression profile by next-generation sequencing in a novel rat model of contrast-induced acute kidney injury. Ann Transl Med. 2019;7(8):178.

Fang Y, Su Y, Xu J, et al. Varicocele-mediated male infertility: from the perspective of testicular immunity and inflammation. Front Immunol. 2021;12:729539.

Belardin L, Camargo M, Intasqui P, Antoniassi M, Fainetto R, Bertolla R. Cysteine-rich secretory protein 3: inflammation role in adult varicocele. Andrology. 2019;7(1):53-61.

Sañin Z, Celik-Ozenci A, Akkoypulu G, et al. Increased expression of interleukin-1α and interleukin-1β is associated with experimental varicocele. Fertil Steril. 2006;85(suppl 1):1265-1275.

Antonuccio P, Micali AG, Romeo C, et al. NLRP3 Inflammasome: a new pharmacological target for reducing testicular damage associated with Varicocele. Int J Mol Sci. 2021;22(3):1319.

Camargo M, Ibrahim E, Intasqui P, et al. Seminal inflammasome activity in the adult varicocele. Hum Fertil (Camb). 2021;1-15.

Micheli L, Collodel G, Cerretani D, et al. Relationships between ghrelin and estestet with MDA, proinflammatory cytokines, GSH/-GSSG ratio, catalase activity, and semen parameters in infertile patients with leukocytespermia and varicocele. Oxid Med Cell Longev. 2019;2019:7261842.

Zeinali M, Hadian Amree A, Khorramdelazad H, Karaimi H, Abedizadeh M. Inflammatory and anti-inflammatory cytokines in the seminal plasma of infertile men suffering from varicocele. Andrologia. 2017;49(6):e12685.

Ata-Abadi NS, Mowla SJ, Aboutalebi F, et al. Hypoxia-related long noncoding RNAs are associated with varicocele-related male infertility. PLoS One. 2020;15(4):e0232357.

Asghafzadeh AM, Sadighi Gilani MA, Topraggaleh TR, et al. Oxidative stress-related miRNAs in spermatozoa may reveal the severity of damage in grade III varicocele. Andrologia. 2020;52(9):e13598.

Zhao L, Zhu Z, Yao C, et al. VEGFC/VEGFR3 signaling regulates mouse Spermatogonial cell proliferation via the activation of AKT/ MAPK and cycin D1 pathway and mediates the apoptosis by affecting caspase 3/9 and Bcl-2. Cell Cycle. 2018;17(2):225-239.

Huang J, Fang L, Zhang S, Zhang Y, Ou K, Wang C. Long-term exposure to environmental levels of phenanthrene disrupts spermatogenesis in male mice. Environ Pollut. 2021;285:117488.

Yang T, Yang W. The dynamics and regulation of microfilament during spermatogenesis in male mice. Andrology. 2020;744:144635.

Aquilla S, Sisci D, Gentile M, et al. Estrogen receptor (ERα) and ERβ are both expressed in human ejaculated spermatozoa: evidence of their direct interaction with phosphatidylinositol-3'-OH kinase/Akt pathway. J Clin Endocrinol Metabol. 2004;89(14):1443-1451.

Dube E, Dufresne J, Chan PTK, Cyr DG. Epidermal growth factor regulates connexin 43 in the human epididymis: role of gap junctions in azoospermia. Hum Reprod. 2012;27(8):2285-2296.

Wang D, Zhao W, Liu J, et al. Effects of HIF-1α on spermatogenesis of varicocele rats by regulating VEGF/Pi3K/Akt signaling pathway. Reprod Sci. 2021;28(4):1161-1174.
46. Zhao J, Ma W, Zhong Y, et al. Transcriptional inhibition of lncRNA gadd7 by CRISPR/dCas9-KRAB protects spermatocyte viability. *Front Mol Biosci*. 2021;8:652392.

47. Zhao J, Li H, Deng H, et al. LncRNA gadd7, increased in varicocele patients, suppresses cell proliferation and promotes cell apoptosis. *Oncotarget*. 2018;9(4):5105-5110.

48. D’Andrea MR, Alicknavitch M, Nagele RG, Damiano BP. Simultaneous PCNA and TUNEL labeling for testicular toxicity evaluation suggests that detection of apoptosis may be more sensitive than proliferation. *Biotech Histochem*. 2010;85(3):195-204.

49. Liu X, Wang Z, Liu F. Chronic exposure of BPA impairs male germ cell proliferation and induces lower sperm quality in male mice. *Chemosphere*. 2021;262:127880.

50. Agostinho A, Manneberg O, van Schendel R, et al. High density of REC8 constrains sister chromatid axes and prevents illegitimate synaptonemal complex formation. *EMBO Rep*. 2016;17(6):901-913.

**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

How to cite this article: Wang S, Kang J, Song Y, et al. Long noncoding RNAs regulated spermatogenesis in varicocele-induced spermatogenic dysfunction. *Cell Prolif*. 2022;55(5):e13220. doi:10.1111/cpr.13220