



RESEARCH ARTICLE

miR-21-5p ameliorates Busulfan-induced testicular dysfunction and maintains spermatogenesis

Mengfei Zhang^{1*}, Shicheng Wan^{1*}, Wenbo Chen¹, Donghui Yang¹, Congliang Wang¹, Balun Li¹, Aierken Aili^{1,4}, Xiaomin Du¹, Yunxiang Li¹, Wenping Wu¹, Yuqi Wang¹, Fangde Xie¹, Xuan Luo¹, Na Li^{1,3}, Xueling Li², Lei Yang², Ahmed Hamed Arisha^{5,6}, Jinlian Hua^{1,3#}

¹ College of Veterinary Medicine, Shaanxi Centre of Stem Cells Engineering & Technology, Northwest A&F University, Yangling 712100, China

² Key Laboratory for Mammalian Reproductive Biology and Biotechnology, Ministry of Education/Inner Mongolia University, Hohhot 010021, China

³ Key Laboratory of Livestock Biology, Northwest A&F University, Yangling 712100, China

⁴ State Key Laboratory on Pathogenesis, Prevention and Treatment of High Incidence Diseases in Central Asia, Xinjiang Medical University, Urumqi 830011, China

⁵ Department of Animal Physiology and Biochemistry, Faculty of Veterinary Medicine, Badr University in Cairo, Badry 11829, Egypt

⁶ Department of Physiology and Laboratory of Biotechnology, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44511, Egypt

Highlights

- miR-21-5p is crucial for the self-renewal and maintenance of SSCs in both goat and mouse.
- The overexpression of miR-21-5p *in vivo* can ameliorate Busulfan-induced testicular dysfunction, and maintained spermatogenesis.
- SPRY1 and FASLG are targets of miR-21-5p, thereby activating downstream signaling pathways such as MAPK/ERK, PI3K-AKT, and apoptosis.

Abstract

Spermatogonial stem cells (SSCs) are the key to maintaining production of the sperms and healthy offsprings, and also treating breeding livestock's reproductive damage and infertility. microRNAs act a decisive role in regulating gene expression in many cells and tissues, including in processes such as proliferation, self-renewal, differentiation, and apoptosis of stem cells. However, the miRNA mechanism in regulation of SSCs is still unclear. Here, high-throughput sequencing was used to identify specific miRNAs. We confirmed that miR-21-5p was concentrated in both goat and mouse SSCs, and enhanced the proliferation and antiapoptotic ability of SSCs. *In vivo* experiments

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Mengfei Zhang, E-mail: zmf2049@163.com; #Correspondence Jinlian Hua, Tel: +86-29-87091117, E-mail: jinlianhua@nwsuaf.edu.cn

* These authors contributed equally to this study.

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have shown that miR-21-5p resisted the damage of the chemotherapy drug Busulfan to germ cells, ameliorated Busulfan-induced testicular dysfunction, and maintained spermatogenesis. Further RNA-seq and target gene prediction revealed that *SPRY1* and *FASLG* are targets of miR-21-5p, thereby activating downstream signaling pathways such as MAPK/ERK, PI3K-AKT, and apoptosis. In summary, miR-21-5p is crucial for the self-renewal and maintenance of SSCs. This study provides new avenues for treating breeding livestock's reproductive damages, infertility, oligospermia, and other conditions.

Keywords: miR-21-5p, spermatogonial stem cells, infertility, Busulfan, apoptosis

1. Introduction

The decline of sperm quality and male infertility have become a concern internationally, with an incidence rate of 15% (Ma *et al.* 2016; Punab *et al.* 2017). Spermatogonial stem cells (SSCs) survive in the marginal basement membrane of the seminiferous tubules of male animal testes. SSCs are powerful stem cells that can sustain male animal spermatogenesis and produce sperm, ultimately transmitting genetic information from parents to children (Huang *et al.* 2020). SSCs are particularly useful in genetic inheritance, livestock breeding, and human reproduction, and are crucial for treating reproductive damage and infertility (Yin *et al.* 2021). Thus, SSCs have great application prospects in reproductive and regenerative medicine. The maintenance of SSCs depends on their ability to self-renew, including proliferation, differentiation, and apoptosis (Jin *et al.* 2023). The process of spermatogenesis is complex, involving many endocrine and paracrine signals within the body and tissue cells to regulate the self-renewal of SSCs and the differentiation of spermatocytes, leading to mitosis and two subsequent meiotic divisions, and spermatogenesis to produce sperm. Each testicle contains approximately 35,000 SSCs, accounting for approximately 0.03% of all cells in testes, and thus, the separation and purification of SSCs are difficult and inefficient (Du *et al.* 2021). Transcription factors, cytokines, and miRNAs play a decisive role in the fate of SSCs (Yu *et al.* 2014). Therefore, studying the developmental mechanism of spermatogonial stem cells is important in finding targets for treating male infertility.

In recent years, high-throughput sequencing technology has been efficient and sensitive in detecting miRNA expression, which is very helpful for studying specific miRNAs in spermatogonial stem cells (Thum *et al.* 2008). Researchers used high-throughput sequencing to analyze the differential expression of miRNA in the Thy1+ testicular cell population concentrated with SSCs and

the Thy1- cell population mainly composed of testicular somatic cells (Niu *et al.* 2011). Sequencing analysis revealed that miR-21-5p, as well as miR-34c, miR-183, and miR-146a were efficiently expressed in Thy1-positive SSC enriched populations compared to Thy1-negative somatic cells. Short-term inhibition of miR-21-5p in the SSC-rich reproductive cell culture increased the number of apoptotic cells and obviously reduced the number of donor-derived colonies formed by transplanting SSCs in the recipient testis, showing the significance of miR-21-5p in maintaining SSC self-renewal (Niu *et al.* 2011). Our previous research found that miR-21 may be a marker of SSCs (Wu *et al.* 2014). However, the specific regulatory mechanism and downstream target genes of miRNAs are still unclear, and require further research.

Therefore, it is of great significance to explore the miRNAs involved in the self-renewal regulation of SSCs, study the target genes of miRNAs, and investigate the function of miRNAs in disease treatment. Here, we confirm that miR-21-5p is extremely enriched in both goat and mouse SSCs and that miR-21-5p enhances the resistance to apoptosis of SSCs to apoptosis. Further transcriptome sequencing revealed that *SPRY1* and *FASLG* were downstream target genes, thereby activating downstream signaling pathways and inhibiting cell apoptosis. This discovery expands the mechanism of miRNA regulation of spermatogonial stem cells, providing new methods for treating reproductive damage, infertility, oligospermia, and other conditions.

2. Materials and methods

2.1. Animals and sample gathering

The testicular tissue of 3-mon-old Guanzhong dairy goats was used to isolate spermatogonial stem cells and somatic cells. Finally, these cells were used for high-throughput sequencing and gene editing. All male mice used in the reproductive defect model of mice were purchased from Chengdu Dashuo Experimental Animal Co., Ltd., China. Mice grow in an environment

without specific pathogens, under 12 h of light and 12 h of darkness. In the experiment, each group had at least three individuals. Mouse model of reproductive damage was caused by chemotherapy drugs. MiR-NC and miR-21-5p lentivirus were injected into seminiferous tubules of 8-wk-old healthy mice, respectively. After 5 d, mice were treated with 30 mg kg⁻¹ busulfan for chemotherapy. Samples were taken 3 and 20 d after chemotherapy to detect fertility and testicular morphology (Goodyear and Brinster 2017).

2.2. Cell isolation and culture

All primary cells and cell lines in this study were cultured in a constant temperature incubator (Thermo, USA) at 37°C and 5% CO₂, and the medium was changed once a day. Stable cell lines HEK293T cells and GC1-SPG cells were maintained in DMEM medium. Male germ stem cells of goat (mGSCs) used in our study were stored at Stem Cell Center of Shaanxi. Lipo2000 (Beyotime, China) was used for vector or miRNA transfection following product manual. We first collected the testicles of 3-mon-old Guanzhong dairy goats, and then cut the testicular tissue into fragments, which were digested into individual cells using enzymes (Zhu *et al.* 2013). And then we used 40 µm copper to filter suspension of individual cells (Kou *et al.* 2023). Afterwards, we purified these individual cell suspensions using MACS technology to obtain CD49f-positive spermatogonial stem cells. CD49f-positive SSCs of dairy goats were cultured in DMEM/F12 medium, which contained 1% FBS, 10% KSR, etc. (Du *et al.* 2021).

2.3. qRT-PCR

We used TRIzol reagent (TaKaRa) to extract total RNA from testicles and different cells. Used reverse transcription kit (Thermo) to synthesize cDNA for total RNA. Performed qRT-PCR analysis using SYBR reagent (Vazyme) on CFX96 instrument (BioRad, CA, USA). The final mRNA expression level was standardized to *GAPDH*. Appendix A displayed all the primers used in this study (Zhang *et al.* 2023).

2.4. Immunofluorescence, EdU and TUNEL staining

Method for immunofluorescence, EdU and TUNEL staining referred to kit manual, which included EdU Kit (RiboBio, China) and TUNEL Kit (Beyotime, C1089, China) (Li *et al.* 2019). Primary antibodies used in staining: anti-CD49f (1:100, BioLegend, San Diego, CA), anti-ZBTB16 (1:100, Bioss, bs-5971R, China), anti-OCT4

antibody (1:200, abcam), anti-STAR (1:200, Proteintech, 67130-1-Ig, China). Secondary antibodies: Alexa Fluor 488 (1:400, ZSGB-BIO, ZF-0511, China), Alexa Fluor 594 (1:400, ZSGB-BIO, ZF-0513, China), and DAPI (Beyotime, C1002, China).

2.5. Western blot

Our previous research has reported detailed experimental methods for WB (Wei *et al.* 2021; Yang 2023). Primary antibodies: anti-SPRY1 (Immunoway, USA), anti-beta Actin (Proteintech, China), anti-P53 (Proteintech, China), anti-p-ERK1/2 (Immunoway, USA), anti-BCL2 (Proteintech, China), anti-Caspase 9 (Proteintech, China), anti-Caspase 3 (CST, USA), anti-PI3K (Immunoway, USA), anti-AKT (Immunoway, USA), anti-p-AKT (Immunoway, USA), anti-Cyclin E (Proteintech, China), anti-ERK1/2 (Immunoway, USA), anti-P21 (Proteintech, China), anti-FASLG (Santa, USA), anti-RHOB (Proteintech, China). Secondary antibodies: anti-mouse IgG-HRP (Boster, USA), anti-rabbit IgG-HRP (Boster, USA). We used ImageJ software to determine the values of bands in WB (Yu *et al.* 2022).

2.6. Dual-luciferase reporter assay

To analyze the regulatory effects of miR-21-5p on *SPRY1* and *FASLG* genes, we obtained luciferase reporter vectors containing WT-SPRY1/FASLG 3'UTR and MUT-SPRY1/FASLG 3'UTR vectors. A dual-luciferase reporter system (Beyotime, China) was used to evaluate promoter activity according to manufacturer's instructions. According to kit instructions, we used the constructed dual luciferase reporting system and kit (Beyotime, China) to evaluate promoter activity.

2.7. RNA-seq and data analysis

Firstly, Trizol reagent (TaKaRa) was used to isolate total RNA from different cell samples, followed by the generation of RNA Seq library (Zhang *et al.* 2023). Then, RNA-seq was performed by Beijing GENOMICS INSTITUTE, Ltd., China on Illumina sequencer. Mapped clean reads to *Capra hircus* reference genome GCF_001704415.1. Using RPKM screening of genes to obtain DEGs. Finally, GO and KEGG were used to enrich the functions of DEGs (Wu *et al.* 2021).

2.8. Cell growth curve

Measurement of cell growth curve and cell population doubling time (PDT): Different cell lines were inoculated

into 24-well culture plate, which was recorded as d 0. Continuously changed the culture medium for these cells, and counted these cells in three wells every 24 h. Counted continuously for 5 d. Drew cell growth curve based on the initial number of inoculated cells and the number of cells per day (Zhang *et al.* 2021).

2.9. Flow cytometry

We used AnnexinV-FITC Kit (Beyotime, C1062S, China) for apoptosis detection and Cell Cycle Kit (Beyotime, C1052, China) for cell cycle detection. We inoculated GC1-SPG or mGSCs into 6-well culture plates. Subsequently, we transfected cells with miR-NC, miR-21-5p-inhibitor or miR-21-5p-mimic. After 48 h of treatment, cells were digested and collected with 0.25% trypsin, and these cells were used for cell cycle detection. If transfected cells were not digested and treated separately with or without Busulfan, and next these cells were collected 24 h later for apoptosis detection (Ries *et al.* 2023; Sadeghi and Wright 2023).

2.10. CCK8

We used CCK8 Reagent Kit (Beyotime, C0038, China) to detect cell viability. Firstly, we inoculated GC1-SPG or mGSCs into 96-well plates. Ensured that there were 2,000 cells in each well at the beginning of the experiment. Next, these cells were subjected to different treatments, such as transfection with different miRNA treatments or addition of different concentrations of Busulfan. After the processing time was over, discard the culture medium and added an equal volume of CCK8 reagent to each well. After waiting for 1 h in constant temperature incubator, measured the absorbance of 450 nm (Aierken *et al.* 2022).

2.11. Statistical analyses

All statistical analyses were conducted using GraphPad Prism (v9.0). All data were reported as mean±SD. Each treatment had at least three replicates. Paired Student's *t*-test or ANOVA analysis was used for obtaining *P*-value (ns, *P*>0.05; *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001).

3. Results

3.1. Identification of miR-21-5p as a marker miRNA of spermatogonial stem cells

SSCs are the foundation of spermatogenesis and are valuable in the study of male reproduction. Our laboratory

has previously found that CD49f serves as a marker protein for sorting SSCs (Wu *et al.* 2014; Du *et al.* 2021). To search for miRNAs specifically expressed in SSCs, CD49f magnetic bead antibodies was used to purify CD49f-positive SSCs and negative other somatic cells from goat testicular cells through magnetic cell sorting technology (MACS) (Fig. 1-A and B). Subsequent tests found that the CD49f-positive cell population had higher expression levels of *CD49f* and *Zbtb16* compared to these negative cells, which are known marker genes for SSCs, while the expression levels of these Sertoli cell marker genes *Sox9* were lower (Fig. 1-C and D).

Illumina high-throughput sequencing technology provided in-depth analysis of miRNA expression in different cell groups. Goat CD49f-positive SSCs showed high expression of miR-10b, miR-23a, miR-24, miR-21-5p (Wu *et al.* 2014) and other miRNAs (Fig. 1-E). miR-21-5p was also extensively expressed in Thy1-positive SSCs (Fig. 1-E) (Niu *et al.* 2011). Moreover, quantitative detection revealed that miR-21-5p was extensively expressed in both CD49f-positive cells and male goat spermatogonial stem cell lines (mGSCs) (Fig. 1-F). Additionally, miR-21-5p expression level in the testes gradually decreased with increasing goat age (Fig. 1-G). The base sequence analysis of miR-21-5p in various species revealed that miR-21-5p was concentrated conserved in different species (Fig. 1-H), indicating that miR-21-5p was a miRNA specific marker for SSCs.

3.2. miR-21-5p increases the proliferation of SSCs by promoting S-phase

To determine the effect of miR-21-5p in SSCs, we constructed lentiviral expression vectors for the miR-21-5p mimic, miR-21-5p inhibitor, and miR-21-5p NC. Then, goat SSCs (mGSCs) and mouse SSCs (GC1-SPG) stably expressing miR-21-5p-mimic, miR-21-5p-inhibitor, and miR-NC were obtained through lentiviral infection and puromycin screening (Fig. 2-A and C). CCK-8 assays showed that miR-21-5p upregulated the cell viability of SSCs in goats and mice, while inhibiting miR-21-5p obviously inhibited the viability of SSCs (Fig. 2-B and D). The growth curve showed that miR-21-5p significantly increased the cell proliferation rate and shortened the PDT (Fig. 2-E and F). The EdU staining assay demonstrated an increase in the ratio of EdU-positive cells in the miR-21-5p overexpression group and a decrease in the number of EdU-positive cells in the miR-21-5p inhibition group (Fig. 2-G and H). Flow cytometry analysis showed that miR-21-5p increased the percentage of S phase cells, while inhibiting miR-21-5p decreased the percentage of S phase cells (Fig. 2-I and K).

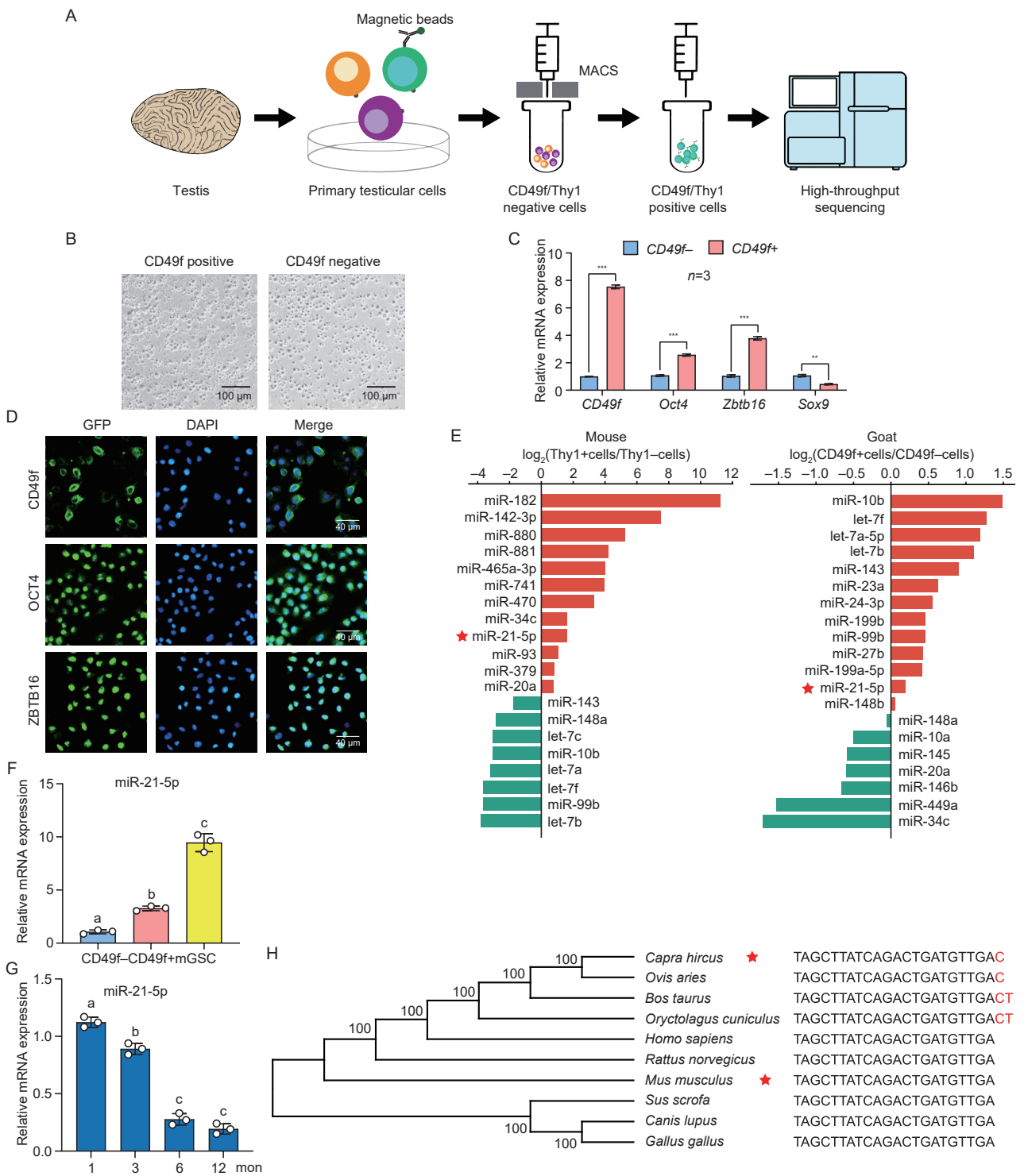


Fig. 1 miR-21-5p highly enriched in spermatogonial stem cells of mice and goats. A, testes were collected and isolated primary testicular cells, then separated CD49f or Thy1 positive spermatogonial stem cells (SSCs) from other CD49f or Thy1 negative cells through MACS. High-throughput sequencing was made to analysis the miRNAs in two types of cells. B, representative images of MACS isolation and concentration of CD49f positive cells in the testicles of dairy goats. C, relative mRNA levels of SSC related genes (*CD49f*, *Oct4*, *Zbtb16*) and Sertoli cell related genes (*Sox9*) in both CD49f positive and negative cell populations. D, detected expression and localization of CD49f, OCT4, and ZBTB16 protein in CD49f positive cells by immunofluorescence staining. The nuclei were stained with DAPI (blue). E, high throughput sequencing analysis of concentrated enriched miRNA groups in mouse (Thy1+) and goat (CD49f+) SSCs. The figure shows the top 20 miRNAs with the highest expression level. F, expression levels of miR-21-5p in CD49f positive cell, CD49f negative cell and mGSC. G, expression levels of miR-21-5p in goat testes of different ages. H, miR-21 phylogenetic tree of different species. Data are mean±SD. **, $P<0.01$; ***, $P<0.001$.

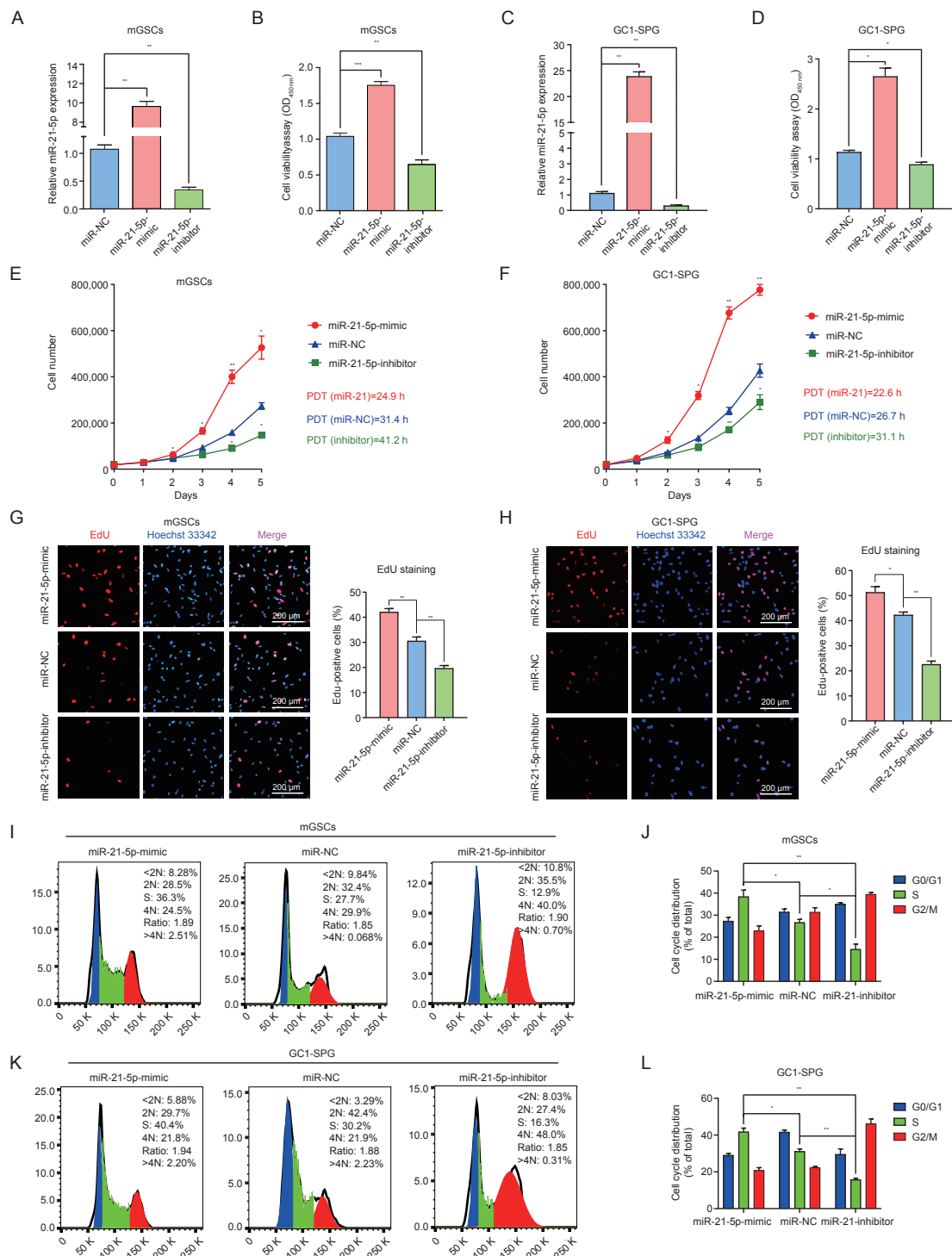


Fig. 2 miR-21-5p stimulated proliferation of spermatogonial stem cells (SSCs) and the increase of S-phase cells in mice and goats. A, expression of miR-21-5p in male germ stem cells of goat (mGSCs) stably expressing miR-NC, miR-21-5p-mimic and miR-21-5p-inhibitor. B, CCK8 detection of cell viability in mGSCs stably expressing miR-21-5p-mimic and miR-21-5p-inhibitor. C, expression of miR-21-5p in GC1-SPG stably expressing miR-21-5p-mimic and miR-21-5p-inhibitor. D, CCK8 detection of cell viability in GC1-SPG stably expressing miR-21-5p-mimic and miR-21-5p-inhibitor. E, growth curve and population doubling time determination of mGSCs stably expressing miR-21-5p-mimic and miR-21-5p-inhibitor. F, growth curve and PDT determination of GC1-SPG stably expressing miR-21-5p-mimic and miR-21-5p-inhibitor. G, EdU staining (left) and quantification (right) of the stably transfected mGSCs. H, EdU staining (left) and quantification (right) of stably transfected GC1-SPG. I, flow cytometry detection of the cell cycle in the stably transfected mGSCs. J, quantification of the cell cycle in mGSCs. K, flow cytometry detection of the cell cycle in the stably transfected GC1-SPG. L, quantification of cell cycle in GC1-SPG. Data are mean±SD. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

3.3. MiR-21-5p enhances the ability of SSCs to resist apoptosis by inhibiting the Caspase family

As a chemotherapy drug, Busulfan often causes apoptosis of SSCs and male infertility (Thirouard *et al.* 2022; Xiong *et al.* 2023). To determine the effect of miR-21-5p on the chemotherapy resistance of SSCs, we treated goat and mouse SSCs stably expressing miR-21-5p-mimic, miR-21-5p-inhibitor, and miR-NC with different concentrations of Busulfan (Fig. 3-A and B). The CCK8 assays results showed that as the concentration of Busulfan increased, the cell viability of SSCs in both goats and mice decreased (Fig. 3-A and B). Compared with that of the group treated with miR-NC and Busulfan, the activity of the miR-21-5p group was obviously increased, while the cell activity of the inhibitor group was significantly reduced (Fig. 3-A and B).

We conducted flow cytometry testing and found that compared with the inhibitor group and the control group, the miR-21-5p group exhibited a tremendous decrease in the apoptosis rate of mGSCs and GC1-SPG cells (Fig. 3-C and E). The detection of apoptotic proteins revealed a substantial increase at the protein level of the apoptotic suppressor BCL2 and a significant decrease in the expression of pro-apoptotic proteins P53, cleaved caspase 3, and cleaved caspase 9 in SSCs overexpressing miR-21-5p (Fig. 3-G and H). In contrast, the expression of P53, cleaved Caspase 3, and cleaved caspase 9 was significantly increased in SSCs that inhibited miR-21-5p (Fig. 3-G and H). In summary, these results indicated that the inhibition of miR-21-5p increased the apoptosis of germ cells and affected the activity of stem cells.

3.4. MiR-21-5p ameliorates busulfan-induced testicular dysfunction and maintains spermatogenesis

To verify whether miR21 could resist the killing effect of the chemotherapy drug busulfan on germ cells and be applied in clinical treatment, we implemented an *in vivo* test as shown in Fig. 4-A. miR-NC and miR-21-5p lentivirus were injected into mouse seminiferous tubules, respectively. After 5 d, the mice were treated with 30 mg kg⁻¹ Busulfan for chemotherapy. Samples were taken 3 and 20 d after chemotherapy to assess fertility and testicular morphology (Fig. 4-A). Subsequently, we collected testes 5 days after busulfan treatment for testing, and TUNEL staining showed that busulfan induced a large amount of germ cell death, but overexpression of miR-21-5p significantly reduced germ cell apoptosis (Fig. 4-B and C). The killing effect of Busulfan on germ cells was greater than that on interstitial cells (Fig. 4-B).

After 20 d of treatment with Busulfan, the testes were tested using histological methods, and HE staining showed that after long-term treatment with Busulfan, almost all germ cells were killed (Fig. 4-D). There were no germ cells in the seminiferous tubules of the miR-NC group, and no sperm in the epididymis, so miR-NC group developed infertility (Fig. 4-D). In the testes of miR-21-5p group, only half of the seminiferous tubules were empty, and the epididymides of miR-21-5p mice were filled with sperm, indicating that miR-21-5p mice could still produce a large number of sperm (Fig. 4-E). The above experiments confirmed that miR-21-5p improved reproductive damage caused by chemotherapy drugs.

3.5. Transcriptome sequencing reveals that miR-21-5p activates ERK and PI3K pathways

To further explore the target genes and downstream signaling pathways regulated by miR-21-5p, we performed transcriptome sequencing of mGSCs stably expressing miR-21-5p-mimic, miR-21-5p-inhibitor, and miR-NC. The sequencing results indicated that 193 genes were upregulated and 277 genes were downregulated in miR-21-5p group (Fig. 5-A). A total of 450 genes were upregulated and 325 genes were downregulated in the inhibitor group (Fig. 5-B). Wayne plot analysis was performed on differentially expressed genes (DEGs), and it was found that 5 DEGs were positively regulated by miR-21-5p, while 19 DEGs were negatively regulated by miR-21-5p (Fig. 5-C).

KEGG analysis of these upregulated and downregulated differentially expressed genes revealed enrichment of KEGG pathways, including PI3K-Akt and MAPK signaling pathways and cell adhesion molecules (Fig. 5-D). Subsequently, GO analysis was performed on these upregulated and downregulated differentially expressed genes, revealing the enrichment of GO pathways, mainly including positive regulation of cell division, cell adhesion, cell migration, chondrocyte proliferation, and positive regulation of glial cell proliferation (Fig. 5-E). The heatmap shows representative genes for positive and negative regulation of miR-21-5p. The genes for negative regulation of miR-21-5p include *SPRY1*, *RHOB*, *TAF9B*, *MAPK10*, *FAS*, *GATA6*, *FGF18*, *MMP9*, etc. The genes for positive regulation of miR-21-5p include *PDCD4*, *ATP5MD*, *COL14A1*, *INSL6*, etc. (Fig. 5-F). Western blotting analysis further showed that overexpression of miR-21-5p inhibited the expression of *SPRY1*, activated the ERK1/2 and PI3K pathways, and raised the level of Cyclin E, reducing P21 expression (Fig. 5-G).

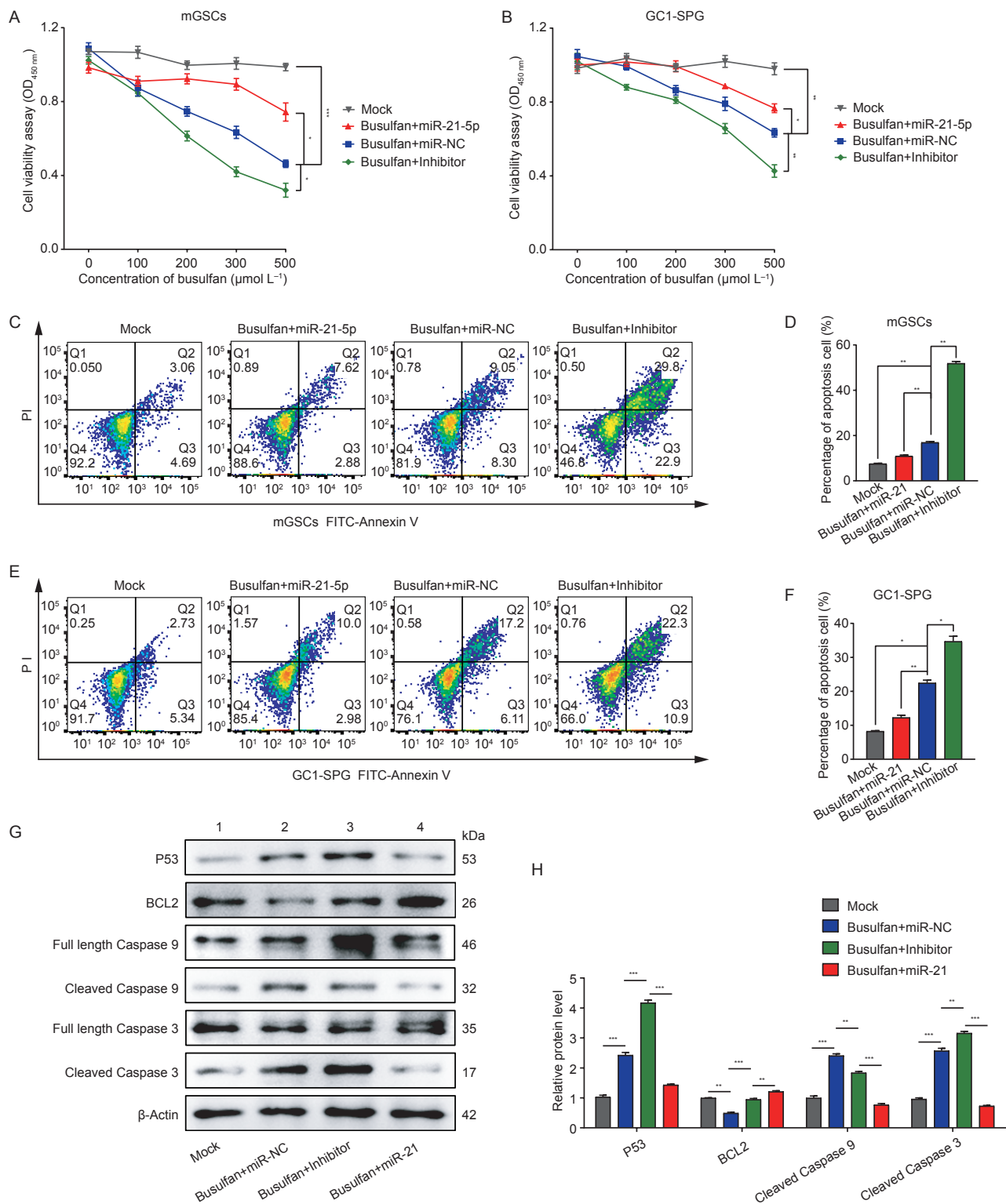


Fig. 3 miR-21-5p decreased sensitiveness of spermatogonial stem cells (SSCs) to busulfan and inhibited apoptosis. A, CCK8 detection of the cell viability in the stably transfected male germ stem cells of goat (mGSCs) after treatment with different doses of busulfan. B, CCK8 detection of the cell viability in the stably transfected GC1-SPG after treatment with different densities of busulfan. C, flow cytometry detection of apoptosis in stable transfected mGSCs after 500 μmol L⁻¹ busulfan treatment. D, statistical analysis of apoptosis in mGSCs. E, detection of apoptosis in stable transfected GC1-SPG after 500 μmol L⁻¹ busulfan treatment. F, statistical analysis of apoptosis in GC1-SPG. G, Western blot detection of the level of apoptosis related proteins in mGSCs stably expressing miR-21-5p-mimic and miR-21-5p-inhibitor. H, quantification of WB results. Data are mean±SD. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

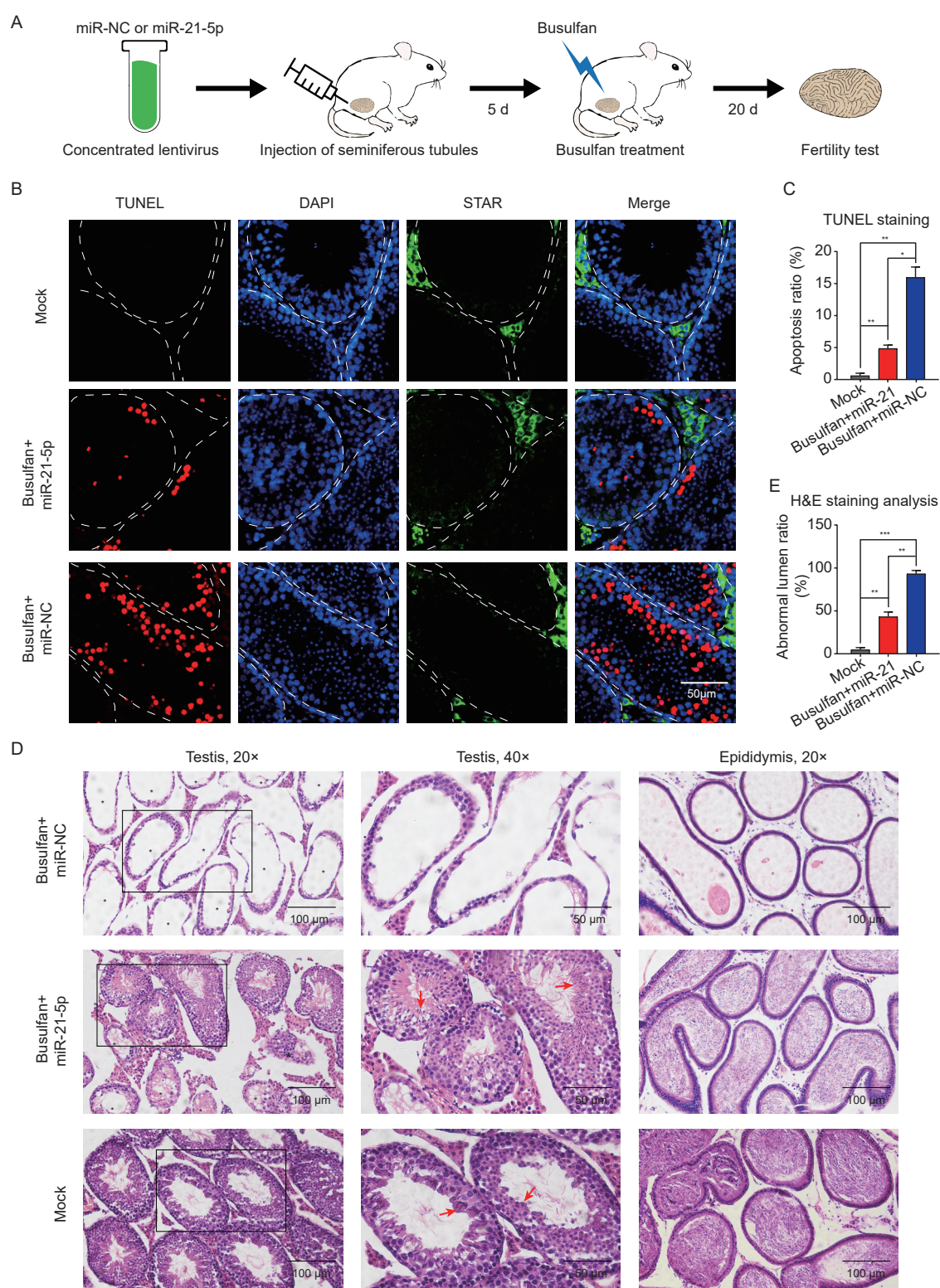


Fig. 4 miR-21-5p ameliorated busulfan-induced testicular dysfunction and maintained spermatogenesis. A, construction of mouse model of chemotherapy drug damage and miR-21-5p treatment. B, TUNEL staining of Mock, busulfan+miR-21-5p and Busulfan+miR-NC testes. STAR represented interstitial cells between lumens. Scale bar: 50 μ m. C, apoptosis ratio in testes for quantification of TUNEL staining. Counted no less than 30 seminiferous tubules. D, H&E-staining of testes (left, middle) and epididymides (right) sections from groups. The middle images were the enlarged result in the black box of the left images. The asterisk marked the empty lumen, and the red arrow indicated mature sperm. E, percentage of abnormal lumens in H&E staining to total lumens. Counted no less than 30 seminiferous tubules. Data are mean $\pm$ SD. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

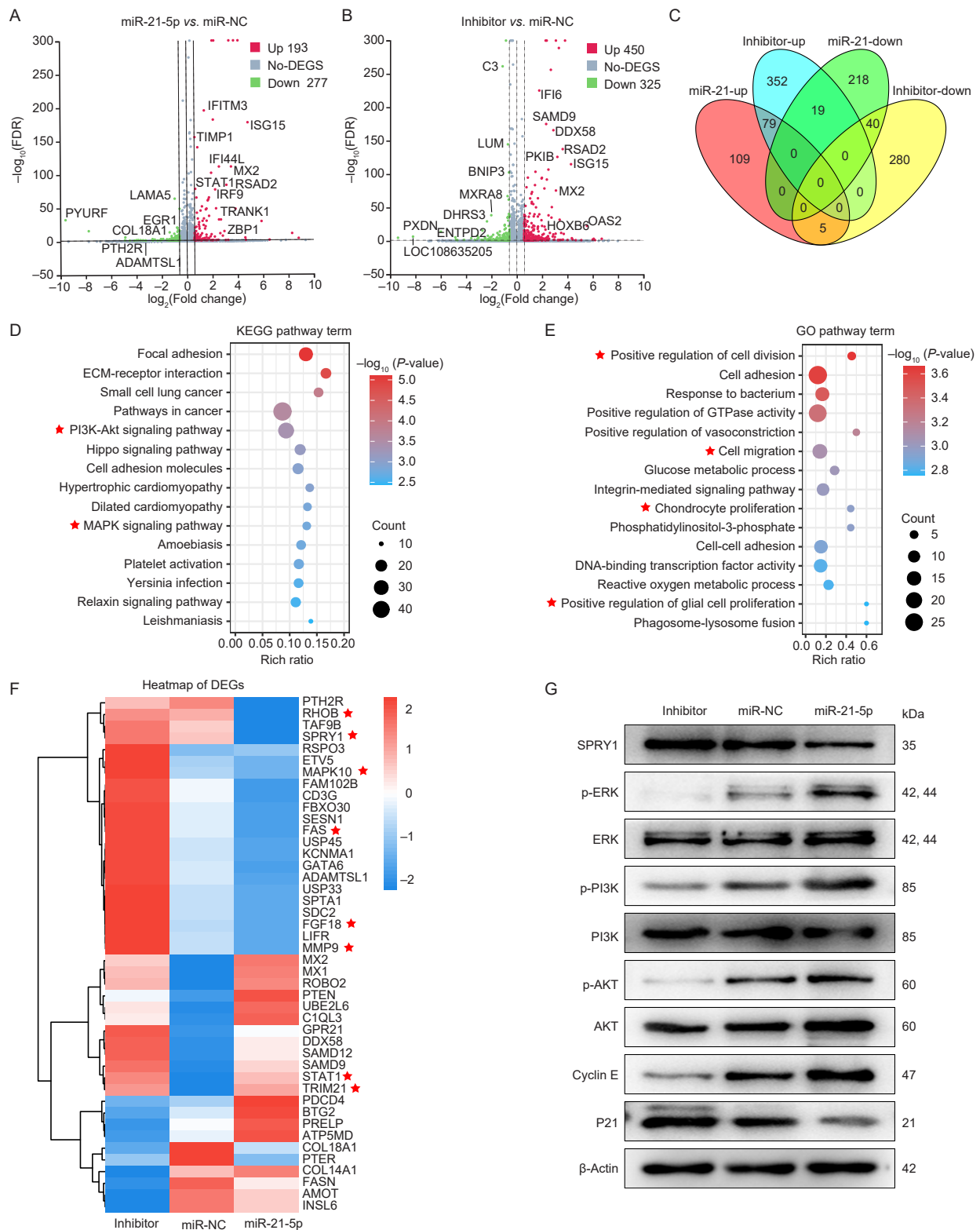


Fig. 5 RNA-seq identified target genes and regulatory pathways of miR-21-5p. A, differentially expressed genes were determined by RNA-seq of male germ stem cells of goat (mGSCs) stably expressing miR-21-5p-mimic using volcano plot. B, volcano plot of DEGs determined by RNA-seq of mGSCs stably expressing miR-21-5p-inhibitor. C, Venn diagram of DEGs co-regulated by miR-21-5p-mimic and miR-21-5p-inhibitor. D, KEGG analysis of differentially expressed genes. E, GO analysis of differentially expressed genes. F, heatmap of RNA-seq data showing representative differential genes regulated by miR-21-5p. G, Western blot detection of downstream target genes and signaling pathway related proteins regulated by miR-21-5p.

3.6. MiR-21-5p increases the proliferation of SSCs by inhibiting SPRY1

Our previous experiment showed that miR-21-5p promoted the proliferation of SSCs, and transcriptome sequencing found that miR-21-5p raised ERK1/2 pathways. To further investigate the regulatory mechanisms involved, we used a joint analysis of TargetScan7.2 and transcriptome sequencing to excavate prospective target genes. We tested approximately 200 possible potential target genes and found that *SPRY1* was a candidate gene (Fig. 6-A). Then, we constructed wild-type *SPRY1* 3'UTR (WT-SPRY1-3'UTR) and mutant *SPRY1* 3'UTR (MUT-SPRY1-3'UTR) vectors for the dual luciferase reporter test (Fig. 6-A). After cotransfection of the two

vectors into SSCs, the dual luciferase activity of the cells cotransfected with the *SPRY1*-3'UTR wild-type vector and miR-21-5p was lower than that of the cells cotransfected with the *SPRY1*-3'UTR wild-type vector and miR-NC (Fig. 6-B). However, we found that dual luciferase level in the cells cotransfected with MUT-*SPRY1*-3'UTR did not seem to be affected (Fig. 6-B). Protein and mRNA levels also showed that inhibition of *SPRY1* and P21 expression in miR-21-5p cells increased the expression of Cyclin E (Fig. 6-C and E). Different quality of miR-21-5p vectors were transfected into SSCs, and it was found that the expression level of *SPRY1* decreased with the increase in miR-21-5p (Fig. 6-D). In summary, the experimental results indicated that miR-21-5p increased the proliferation of SSCs by directly repressing *SPRY1*.

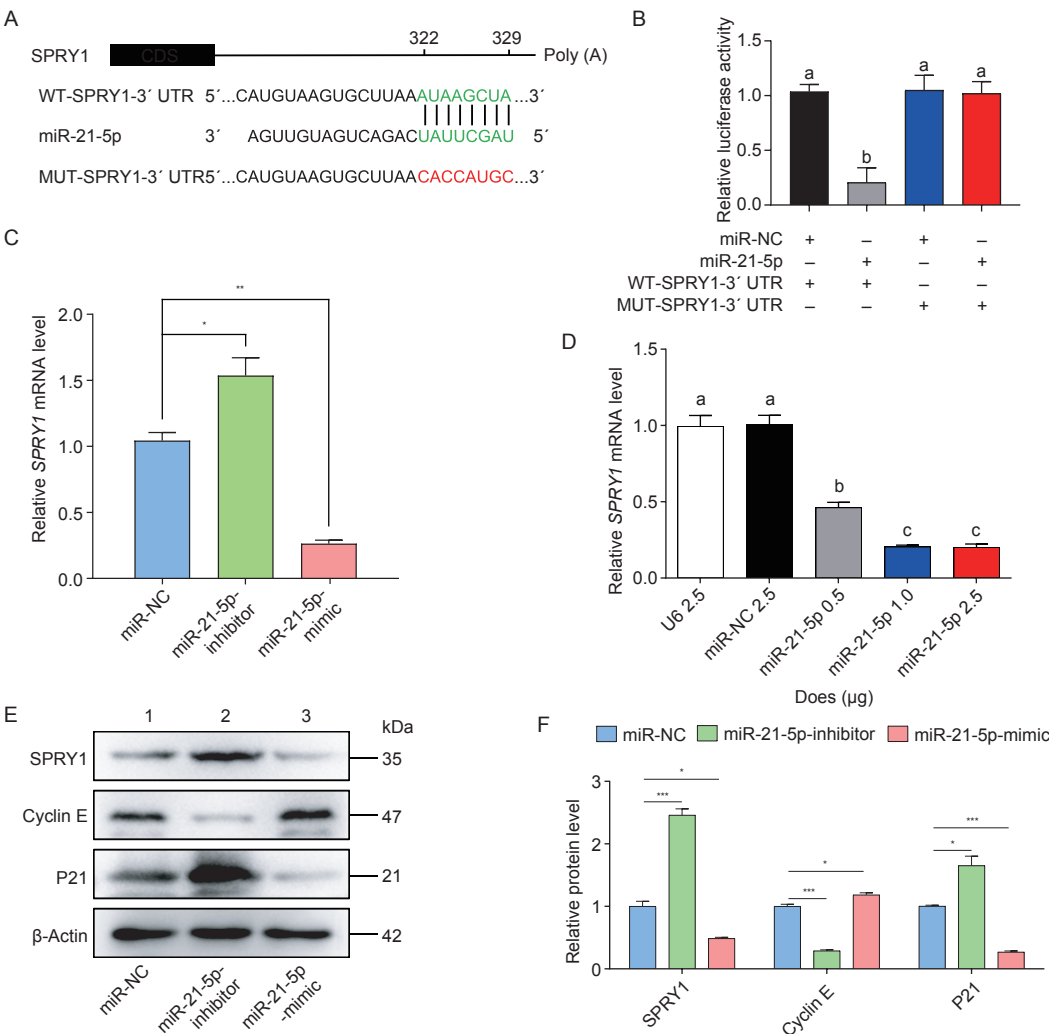


Fig. 6 miR-21-5p raised the proliferation of spermatogonial stem cells (SSCs) by reducing *SPRY1*. **A**, target acids of miR-21-5p and mutation acids in *SPRY1* 3'UTR. **B**, dual luciferase detection of miR-21-5p with wild-type *SPRY1* 3'UTR vectors or miR-21-5p with mutant *SPRY1* 3'UTR vectors. **C**, *SPRY1* mRNA levels after added with miR-NC, miR-21-5p-inhibitor and miR-21-5p-mimic vectors. **D**, *SPRY1* level after transfection with different vectors and doses. **E**, Western blot detection of *SPRY1*, Cyclin E, and P21 proteins in male germ stem cells of goat (mGSCs). **F**, quantification of WB detection results. Data are mean $\pm$ SD. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

3.7. MiR-21-5p suppresses apoptosis of SSCs by targeting FASLG

A previous experiment found that miR-21-5p enhanced the antiapoptotic ability of SSCs. We used TargetScan7.2 and transcriptome sequencing combined analysis to predict potential target genes. Transcriptome sequencing revealed that the apoptosis-related gene *FASLG* was negatively regulated by miR-21-5p, and *FASLG* was one of the candidate target genes for miR-21-5p. Similar to the previous result, dual luciferase reporter vectors (Fig. 7-A) were constructed for analysis. The analysis suggested

that miR-21-5p significantly repressed the dual luciferase level of *FASLG* (Fig. 7-B). *FASLG* significantly decreased the mRNA and protein levels in miR-21-5p group and was prominently enhanced in inhibitor group (Fig. 7-C and E). Different quality miR-21-5p vectors were transfected into SSCs, and we observed that the level of *FASLG* decreased with the increase of miR-21-5p (Fig. 7-D). WB analysis also found that miR-21-5p significantly reduced the levels of *FASLG*, *RHOB*, and cleaved Caspase 3, while the inhibitor group showed the opposite effects (Fig. 7-E and F). Our above experimental results prove that miR-21-5p inhibited the death of SSCs by targeting *FASLG*.

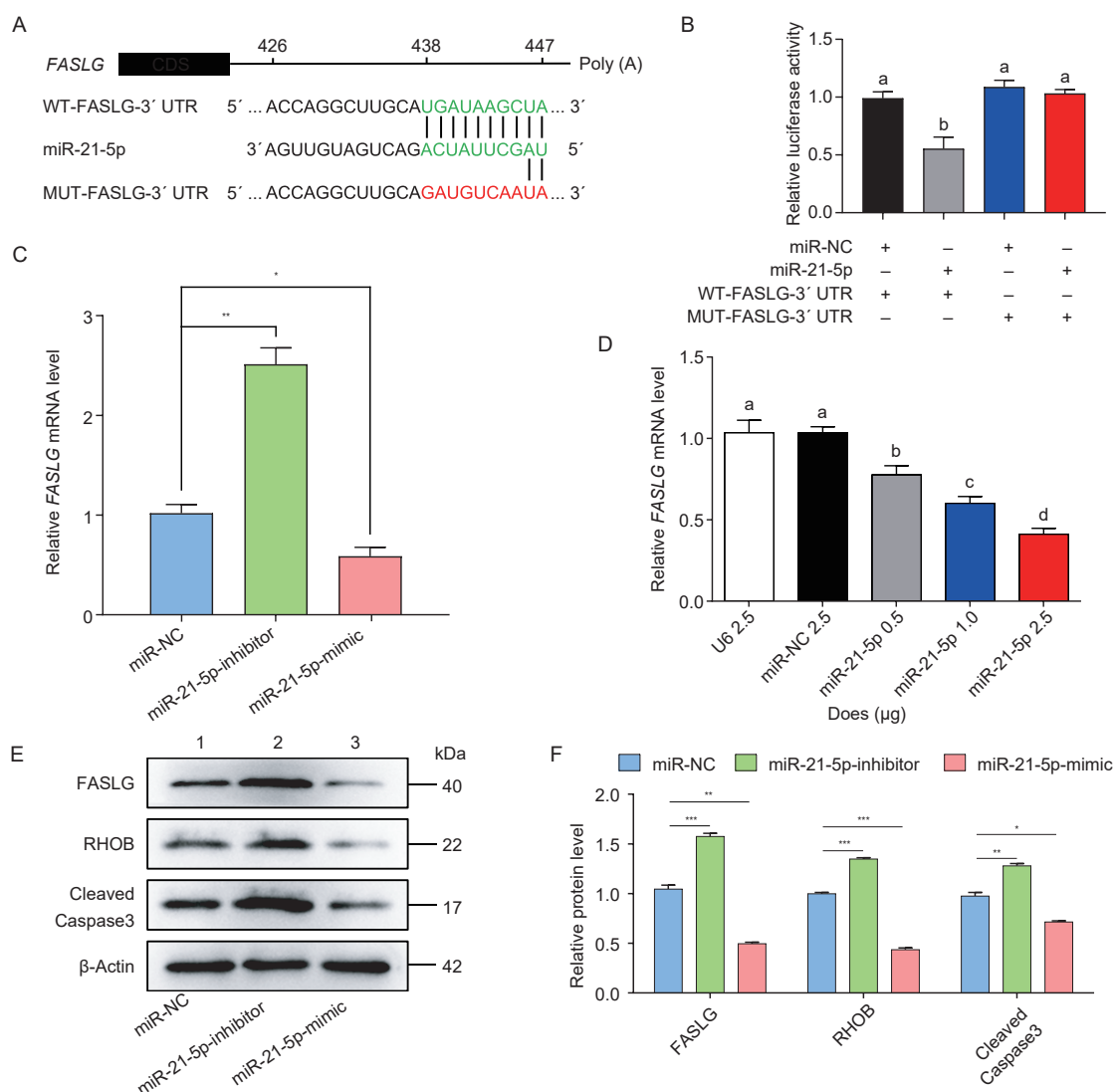


Fig. 7 miR-21-5p restrained apoptosis of spermatogonial stem cells (SSCs) by targeting *FASLG*. A, target acids of miR-21-5p and mutation acids in *FASLG* 3'UTR. B, dual luciferase detection of miR-21-5p with wild-type *FASLG* 3'UTR vectors or miR-21-5p with mutant *FASLG* 3'UTR vectors. C, *FASLG* mRNA levels after adding with miR-NC, miR-21-5p-inhibitor and miR-21-5p-mimic vectors. D, *FASLG* expression level after transfection with different vectors and doses. E, Western blot detection of *FASLG*, *RHOB*, and cleaved Caspase3 proteins in male germ stem cells of goat (mGSCs). F, quantification of WB detection results. Data are mean±SD. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

4. Discussion

Our study revealed that miR-21-5p was concentrated in SSCs. miR-21-5p inhibited *SPRY1* activity, participated in signaling pathways such as MAPK/ERK and PI3K-AKT, increased Cyclin E expression, inhibited P21 expression, promoted the cell cycle, and accelerated cell proliferation of SSCs (Fig. 8). Moreover, miR-21-5p inhibited *FASLG* to reduce the expression of RHOB and P53, thereby increasing the level of BCL2, reducing the expression of cleaved caspase 3/9, inhibiting cell apoptosis, and decreasing the sensitivity of SSCs to Busulfan (Fig. 8). In summary, miR-21-5p plays an important role in self-renewal and proliferation of SSCs. miR-21-5p ameliorated Busulfan-induced testicular dysfunction and maintained spermatogenesis.

In this study, we first analyzed the enriched miRNAs in goat and mouse SSCs through high-throughput sequencing and found that miR-21-5p was concentrated in both goat and mouse SSCs. Our analysis of the sequence of miR-21-5p showed that it was almost identical in different species, indicating the importance of this miRNA. Other different miRNAs represent species differences between goats and mice, indicating that the SSCs of the two species have both the same and different

miRNAs (Sheedy et al. 2010). There are many related studies on other miRNAs. MiR-125b overexpression led to incremental rates of pregnancy loss in mice (Sun et al. 2022). miR-34c enhanced mouse SSC differentiation by targeting Nanos2 (Yu et al. 2014; Greenough et al. 2022).

We performed RNA-seq on goat SSCs stably expressing miR-21-5p-mimic, miR-21-5p-inhibitor, and miR-NC and identified a large number of differentially expressed genes. Wayne plot analysis showed that 5 genes were positively regulated by miR-21-5p and 19 genes were negatively regulated. Two key target genes, *SPRY1* and *FASLG* were identified by a joint analysis of transcriptome sequencing and TargetScan7.2. *SPRY1* is a known negative regulatory gene of the MAPK/ERK signaling pathway, and studies have shown that *SPRY1* inhibits phosphorylation and reduces cell proliferation (Hatley et al. 2010; Du et al. 2019). miR-21-5p could inhibit *SPRY1* and activate signaling pathways such as MAPK/ERK and PI3K-AKT, thereby increasing protein expression levels such as Cyclin E and promoting the proliferation of SSCs (Gu et al. 2022).

FASLG signaling is a crucial part of the cell apoptosis pathway, and this molecule has been identified as a type II transmembrane protein belonging to the tumor necrosis factor (TNF) superfamily (Kang et al. 2022; Wei et al. 2022). This molecule could activate the apoptosis

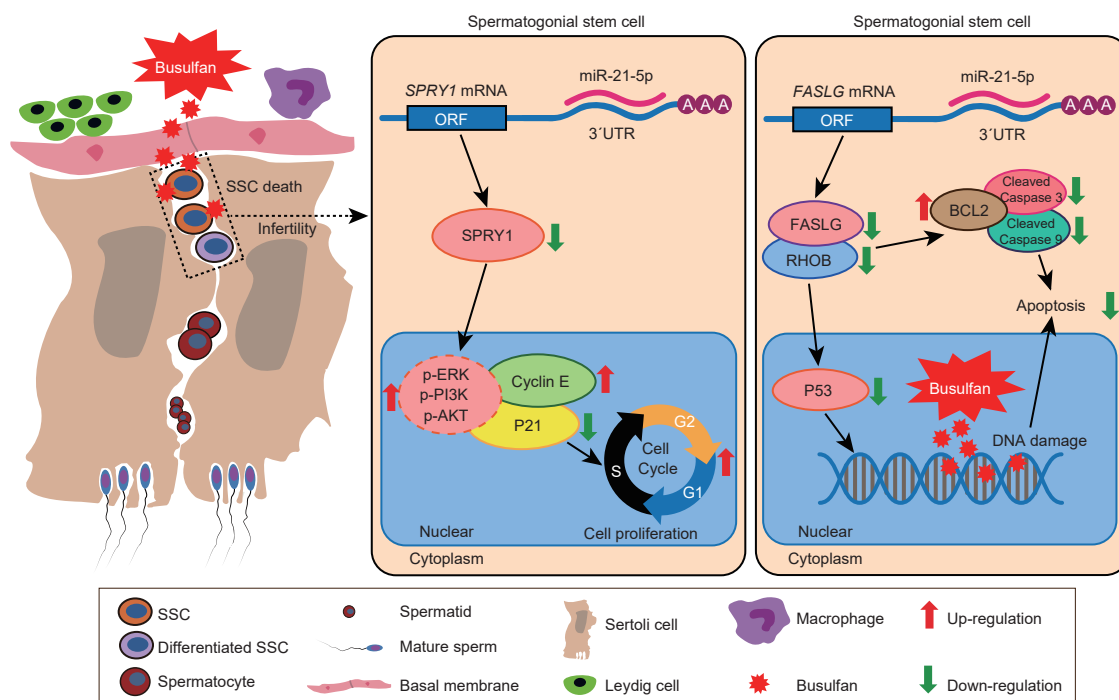


Fig. 8 Pattern diagram of miR-21-5p's role in treatment of testicular dysfunction induced by busulfan. The pattern diagram of miR-21-5p in targeting *SPRY1* and *FASLG*. miR-21-5p resisted damage of chemotherapy drug busulfan to germ cells and ameliorated busulfan-induced testicular dysfunction and maintained spermatogenesis. The red arrow indicated improvement, while the green arrow indicated inhibition.

signaling pathway and cause an increase in the level of cleaved caspase 3/9, thereby inducing cell apoptosis (Zhang *et al.* 2022). In contrast, *FASLG* and *RHOB* caused upregulation of P21 protein, which is a cell cycle suppressor protein that prevents cells from entering the S phase, leading to G1 arrest and inability to proliferate, ultimately leading to apoptosis. miR-21-5p targeted *FASLG* and inhibited its expression, thereby inhibiting the expression of P21, promoting the entry of SSCs into the S phase, and improving their proliferation rate (Jia *et al.* 2017). miR-21-5p also reduced the protein level of cleaved caspase 3/9 through *FASLG*, thereby inhibiting cell apoptosis. *RHOB* is also an important regulatory protein in apoptosis and inflammation pathways. Yang *et al.* (2022) and Miao *et al.* (2021) found that *RHOB* affects colitis by modulating cell signaling and the intestinal microbiome. Our results indicated that the inhibitor of miR-21-5p obviously increased the apoptosis of SSCs, increased their sensitivity to Busulfan, and reduced the proliferation rate of SSCs.

Numerous studies have shown that miR-21 plays an important role in various tissues and organs. Researchers have found that miR-21 participated in regulating liver fibrosis by targeting PTEN signaling and GSH metabolic pathways (Huang *et al.* 2023). Another study found that miR-21 nanoparticles promoted the remote and precise delivery of exogenous miR-21 to the palatal cleft. Then miR-21 accelerated bone formation by upregulating osteogenic markers such as *ALP*, *RUNX2*, and osteoprotegerin and inducing fewer osteoclasts (Liu *et al.* 2023). In terms of inflammatory regulation, endogenous miR-21 activation of *TLR7* in renal epithelial cells contributed to the inflammatory response in renal fibrosis models (Kim *et al.* 2023). Previous studies have reported for the first time that miR-21 played a role in heart disease by affecting ERK-MAP kinase signaling in cardiac fibroblasts. They found that stimuli or programs that induce cardiac stress activated miRNA mediated ERK-MAP kinase activity by affecting *SPRY1*, thereby actively regulating the survival of cardiac fibroblasts, leading to fibrosis, hypertrophy, and cardiac dysfunction. These findings propose new therapeutic entry points for heart disease and demonstrate the broad therapeutic potential of microRNA regulation (Thum *et al.* 2008).

Busulfan is a commonly used drug in chemotherapy that can cause DNA damage and apoptosis in rapidly proliferating cells (Liu *et al.* 2020). Busulfan can kill rapidly proliferating cells, including hair follicle stem cells and spermatogonial stem cells, causing spermatogenesis disorders and male infertility (Morimoto *et al.* 2021; Li *et al.* 2023). After the end of chemotherapy, infertility remains difficult to treat, but miR-21-5p can significantly inhibit the sensitivity of spermatogonial stem cells to

Busulfan, making it an effective method for treating reproductive damage after chemotherapy.

5. Conclusion

In summary, our results indicated that miR-21-5p was concentrated in SSCs and promoted the proliferation of SSCs by targeting *SPRY1* to activate p-ERK1/2, p-PI3K and p-AKT. Moreover, miR-21-5p inhibited apoptosis of SSCs by suppressing *RHOB* and *FASLG*, decreasing sensitivity to Busulfan. Animal experiments have shown that miR-21-5p resisted the damage of chemotherapy drug Busulfan to germ cells, ameliorated Busulfan-induced testicular dysfunction, and maintained spermatogenesis. The results presented here provide valuable insights for regulating the function of SSCs, spermatogenesis, and treatment of reproductive damage.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Ethical approval

All animal experiments in this study were approved by the Animal Care and Use Center of the Northwest A&F University, China (201705A299). Guanzhong dairy goats and C57BL/6J mice of different ages were raised and standardized for management at the Experimental Animal Center of Northwest A&F University, China.

Data availability

The raw sequencing reads of this study have been deposited in NCBI Sequence Read Archive (SRA) database under PRJNA1004872.

Appendix associated with this study is available at <https://doi.org/10.1016/j.jia.2024.02.004>

References

- Aierken A, Li B, Liu P, Cheng X, Kou Z, Tan N, Zhang M, Yu S, Shen Q, Du X, Enkhbaatar B B, Zhang J, Zhang R, Wu X, Wang R, He X, Li N, Peng S, Jia W, Wang C, et al. 2022. Melatonin treatment improves human umbilical cord mesenchymal stem cell therapy in a mouse model of type II diabetes mellitus via the PI3K/AKT signaling pathway. *Stem Cell Research & Therapy*, **13**, 164.
- Du X, Hong L, Sun L, Sang H, Qian A, Li W, Zhuang H, Liang H, Song D, Li C, Wang W, Li X. 2019. miR-21 induces endothelial progenitor cells proliferation and angiogenesis via targeting FASLG and is a potential prognostic marker in deep venous thrombosis. *Journal of Translational Medicine*, **17**, 270.
- Du X, Wu S, Wei Y, Yu X, Ma F, Zhai Y, Yang D, Zhang M, Liu W, Zhu H, Wu J, Liao M, Li N, Bai C, Li G, Hua J. 2021. PAX7 promotes CD49f-positive dairy goat spermatogonial stem cells' self-renewal. *Journal of Cellular Physiology*, **236**, 1481–1493.
- Goodyear S, Brinster R. 2017. Spermatogonial stem cell transplantation to the testis. *Cold Spring Harbor Protocols*, **4**, 094235.
- Greenough M A, Lane D J R, Balez R, Anastacio H T D, Zeng Z, Ganio K, Mcdevitt C A, Acevedo K, Belaidi A A, Koistinaho J, Ooi L, Ayton S, Bush A I. 2022. Selective ferroptosis vulnerability due to familial Alzheimer's disease presenilin mutations. *Cell Death and Differentiation*, **29**, 2123–2136.
- Gu J, Huang W, Wang X, Zhang J, Tao T, Zheng Y, Liu S, Yang J, Chen Z S, Cai C Y, Li J, Wang H, Fan Y. 2022. Hsa-miR-3178/RhoB/PI3K/Akt, a novel signaling pathway regulates ABC transporters to reverse gemcitabine resistance in pancreatic cancer. *Molecular Cancer*, **21**, 112.
- Hatley M E, Patrick D M, Garcia M R, Richardson J A, Bassel-Duby R, Van Rooij E, Olson E N. 2010. Modulation of K-Ras-dependent lung tumorigenesis by MicroRNA-21. *Cancer Cell*, **18**, 282–293.
- Huang T, Liu Z, Zheng Y, Feng T, Gao Q, Zeng W. 2020. YTHDF2 promotes spermatogonial adhesion through modulating MMPs decay via m6A/mRNA pathway. *Cell Death & Disease*, **11**, 37.
- Huang Y, Luo W, Chen S, Su H, Zhu W, Wei Y, Qiu Y, Long Y, Shi Y, Wei J. 2023. Isoviteixin alleviates hepatic fibrosis by regulating miR-21-mediated PI3K/Akt signaling and glutathione metabolic pathway: Based on transcriptomics and metabolomics. *Phytomedicine: International Journal of Phytotherapy and Phytopharmacology*, **121**, 155117.
- Jia X, Xu Y, Wu W, Fan Y, Wang G, Zhang T, Su W. 2017. Aroclor1254 disrupts the blood-testis barrier by promoting endocytosis and degradation of junction proteins via p38 MAPK pathway. *Cell Death & Disease*, **8**, e2823.
- Jin C, Wang Z, Li P, Tang J, Jiao T, Li Y, Ou J, Zou D, Li M, Mang X, Liu J, Ma Y, Wu X, Shi J, Chen S, He M, Lu Y, Zhang N, Miao S, Sun F, et al. 2023. Decoding the spermatogonial stem cell niche under physiological and recovery conditions in adult mice and humans. *Science Advances*, **9**, eabq3173.
- Kang J Y, Wen Z, Pan D, Zhang Y, Li Q, Zhong A, Yu X, Wu Y C, Chen Y, Zhang X, Kou P C, Geng J, Wang Y Y, Hua M M, Zong R, Li B, Shi H J, Li D, Fu X D, Li J, et al. 2022. LLPS of FXR1 drives spermiogenesis by activating translation of stored mRNAs. *Science*, **377**, eabj6647.
- Kim J, Ha S, Son M, Kim D, Kim M J, Kim B, Kim D, Chung H Y, Chung K W. 2023. TLR7 activation by miR-21 promotes renal fibrosis by activating the pro-inflammatory signaling pathway in tubule epithelial cells. *Cell Communication and Signaling: CCS*, **21**, 215.
- Kou Z, Li B, Aierken A, Tan N, Li C, Han M, Jing Y, Li N, Zhang S, Peng S, Zhao X, Hua J. 2023. Mesenchymal stem cells pretreated with collagen promote skin wound-healing. *International Journal of Molecular Sciences*, **24**, 8688.
- Li H T, Zhong K, Xia Y F, Song J, Chen X Q, Zhao W, Zeng X H, Chen T X. 2023. Puerarin improves busulfan-induced disruption of spermatogenesis by inhibiting MAPK pathways. *Biomedicine & Pharmacotherapy=Biomedecine & Pharmacotherapie*, **165**, 115231.
- Li N, Ma W, Shen Q, Zhang M, Du Z, Wu C, Niu B, Liu W, Hua J. 2019. Reconstitution of male germline cell specification from mouse embryonic stem cells using defined factors *in vitro*. *Cell Death and Differentiation*, **26**, 2115–2124.
- Liu B, Wang B, Wang Z, Meng Y, Li Y, Li L, Wang J, Zhai M, Liu R, Wei F. 2023. Near-infrared light-controlled MicroRNA-21-loaded upconversion nanoparticles to promote bone formation in the midpalatal suture. *ACS Applied Materials & Interfaces*, **15**, 43503–43514.
- Liu W, Li N, Zhang M, Liu Y, Sun J, Zhang S, Peng S, Hua J. 2020. Eif2s3y regulates the proliferation of spermatogonial stem cells via Wnt6/β-catenin signaling pathway. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, **1867**, 118790.
- Ma W, Li S, Ma S, Jia L, Zhang F, Zhang Y, Zhang J, Wong G, Zhang S, Lu X, Liu M, Yan J, Li W, Qin C, Han D, Qin C, Wang N, Li X, Gao G F. 2016. Zika virus causes testis damage and leads to male infertility in mice. *Cell*, **167**, 1511–1524.
- Miao C, Yu M, Pei G, Ma Z, Zhang L, Yang J, Lv J, Zhang Z S, Keller E T, Yao Z, Wang Q. 2021. An infection-induced RhoB-Beclin 1-Hsp90 complex enhances clearance of uropathogenic Escherichia coli. *Nature Communications*, **12**, 2587.
- Morimoto H, Ogonuki N, Kanatsu-Shinohara M, Matoba S, Ogura A, Shinohara T. 2021. Spermatogonial stem cell transplantation into nonablated mouse recipient testes. *Stem Cell Reports*, **16**, 1832–1844.
- Niu Z, Goodyear S M, Rao S, Wu X, Tobias J W, Avarbock M R, Brinster R L. 2011. MicroRNA-21 regulates the self-renewal of mouse spermatogonial stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 12740–12745.
- Punab M, Poolamets O, Paju P, Vihlajev V, Pomm K, Ladvá R, Korrovits P, Laan M. 2017. Causes of male infertility: A 9-year prospective monocentre study on 1737 patients with reduced

- total sperm counts. *Human Reproduction*, **32**, 18–31.
- Ries A, Slany A, Pirker C, Mader J C, Mejri D, Mohr T, Schelch K, Flehberger D, Maach N, Hashim M, Hoda M A, Dome B, Krupitza G, Berger W, Gerner C, Holzmann K, Grusch M. 2023. Primary and hTERT-transduced mesothelioma-associated fibroblasts but not primary or htert-transduced mesothelial cells stimulate growth of human mesothelioma cells. *Cells*, **12**, 2006.
- Sadeghi L, Wright A P H. 2023. GSK-J4 Inhibition of KDM6B histone demethylase blocks adhesion of mantle cell lymphoma cells to stromal cells by modulating NF- κ B signaling. *Cells*, **12**, 2010.
- Sheedy F J, Palsson-Mcdermott E, Hennessy E J, Martin C, O'leary J J, Ruan Q, Johnson D S, Chen Y, O'Neill L A J. 2010. Negative regulation of TLR4 *via* targeting of the proinflammatory tumor suppressor PDCD4 by the microRNA miR-21. *Nature Immunology*, **11**, 141–147.
- Sun F, Cai H, Tan L, Qin D, Zhang J, Hua J, Fan X, Peng S. 2022. Placenta-specific miR-125b overexpression leads to increased rates of pregnancy loss in mice. *International Journal of Molecular Sciences*, **23**, 943.
- Thirouard L, Holota H, Monrose M, Garcia M, De Haze A, Damon-Soubeyrand C, Renaud Y, Saru J P, Perino A, Schoonjans K, Beaudoin C, Volle D H. 2022. Identification of a crosstalk among TGR5, GLIS2, and TP53 signaling pathways in the control of undifferentiated germ cell homeostasis and chemoresistance. *Advanced Science* (Weinheim, Baden-Wuerttemberg, Germany), **9**, e2200626.
- Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, Galuppo P, Just S, Rottbauer W, Frantz S, Castoldi M, Soutschek J, Koteliansky V, Rosenwald A, Basson M A, Licht J D, Pena J T R, Rouhanifard S H, Muckenthaler M U, Tuschl T, *et al.* 2008. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. *Nature*, **456**, 980–984.
- Wei W, Chen Q, Liu M, Sheng Y, Ouyang Q, Feng W, Yang X, Ding L, Su S, Zhang J, Fang L, Vidal-Puig A, Wang H Y, Chen S. 2022. TRIM24 is an insulin-responsive regulator of P-bodies. *Nature Communications*, **13**, 3972.
- Wei Y D, Du X M, Yang D H, Ma F L, Yu X W, Zhang M F, Li N, Peng S, Liao M Z, Li G P, Bai C L, Liu W S, Hua J L. 2021. Dmrt1 regulates the immune response by repressing the TLR4 signaling pathway in goat male germline stem cells. *Zoological Research*, **42**, 14–27.
- Wu J, Liao M, Zhu H, Kang K, Mu H, Song W, Niu Z, He X, Bai C, Li G, Li X, Hua J. 2014. CD49f-positive testicular cells in Saanen dairy goat were identified as spermatogonia-like cells by miRNA profiling analysis. *Journal of Cellular Biochemistry*, **115**, 1712–1723.
- Wu X L, Zhu Z S, Xiao X, Zhou Z, Yu S, Shen Q Y, Zhang J Q, Yue W, Zhang R, He X, Peng S, Zhang S Q, Li N, Liao M Z, Hua J L. 2021. LIN28A inhibits DUSP family phosphatases and activates MAPK signaling pathway to maintain pluripotency in porcine induced pluripotent stem cells. *Zoological Research*, **42**, 377–388.
- Xiong Y M, Li Y Y, Lv L, Chen X Y, Li X H, Qin Z F. 2023. Postnatal exposure to low-dose tetrabromobisphenol A increases the susceptibility of mammal testes to chemical-induced spermatogenic stress in adulthood. *Environment International*, **171**, 107683.
- Yang J, Pei G, Sun X, Xiao Y, Miao C, Zhou L, Wang B, Yang L, Yu M, Zhang Z S, Keller E T, Yao Z, Wang Q. 2022. RhoB affects colitis through modulating cell signaling and intestinal microbiome. *Microbiome*, **10**, 149.
- Yang J H. 2023. Cytoprotective effect of bambusae caulis in liquamen by blocking oxidative stress in hepatocytes. *Molecules* (Basel, Switzerland), **28**, 5862.
- Yin H, Kang Z, Zhang Y, Gong Y, Liu M, Xue Y, He W, Wang Y, Zhang S, Xu Q, Fu K, Zheng B, Xie J, Zhang J, Wang Y, Lin M, Zhang Y, Feng H, Xin C, Guan Y, *et al.* 2021. HDAC3 controls male fertility through enzyme-independent transcriptional regulation at the meiotic exit of spermatogenesis. *Nucleic Acids Research*, **49**, 5106–5123.
- Yu M, Mu H, Niu Z, Chu Z, Zhu H, Hua J. 2014. miR-34c enhances mouse spermatogonial stem cells differentiation by targeting Nanos2. *Journal of Cellular Biochemistry*, **115**, 232–242.
- Yu S, Shen Q, Zhang R, Wu X, Zhang J, Zhao W, Wu X, Li N, Peng S, Zhang S, Yang F, Hua J. 2022. KDM4C contributes to Trophoblast-like stem cell conversion from Porcine-induced pluripotent stem cells (piPSCs) *via* regulating CDX2. *International Journal of Molecular Sciences*, **23**, 7586.
- Zhang L, Li L, Wang X, Liu H, Zhang Y, Xie T, Zhang H, Li X, Peng T, Sun X, Dai J, Liu J, Wu W, Ye M, Tan W. 2022. Development of a novel PROTAC using the nucleic acid aptamer as a targeting ligand for tumor selective degradation of nucleolin. *Molecular Therapy Nucleic Acids*, **30**, 66–79.
- Zhang M, Li N, Liu W, Du X, Wei Y, Yang D, Zhou Z, Ma F, Peng S, Zhang S, He X, Bai C, Li G, Hua J. 2021. Eif2s3y Promotes the proliferation of spermatogonial stem cells by activating ERK signaling. *Stem Cells International*, **2021**, 6668658.
- Zhang M F, Wan S C, Chen W B, Yang D H, Liu W Q, Li B L, Aierken A, Du X M, Li Y X, Wu W P, Yang X C, Wei Y D, Li N, Peng S, Li X L, Li G P, Hua J L. 2023. Transcription factor Dmrt1 triggers the SPRY1-NF- κ B pathway to maintain testicular immune homeostasis and male fertility. *Zoological Research*, **44**, 505–521.
- Zhu H, Liu C, Li M, Sun J, Song W, Hua J. 2013. Optimization of the conditions of isolation and culture of dairy goat male germline stem cells (mGSC). *Animal Reproduction Science*, **137**, 45–52.