SIRT5 is associated with asthenozoospermia and regulates GC-2 spd cell proliferation and apoptosis

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Abstract. Infertility is a multifactorial condition that affects ~7% of the male population. The mechanism of male infertility primarily involves intrinsic and environmental factors, of which genetic defects are key for its occurrence. Sirtuin 5 (SIRT5) is primarily localized in mitochondria and associated with male reproduction. SIRT5 is downregulated in asthenozoospermic sperm compared with normal sperm. To explore the role of SIRT5 in male reproduction, Cell Counting Kit-8, EdU, wound healing, and apoptosis assayswere performed in GC-2 spd cells (mouse spermatocytes) which revealed that SIRT5 inhibited apoptosis and promoted cell proliferation and migration. Western blotting was performed and the results showed that SIRT5 was involved in the proliferation of GC-2 spd cells by regulating the PI3K/AKT signaling pathway. The present study demonstrated a mechanism of male infertility, which may aid in its treatment.

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Abbreviations: NAD, nicotinamide adenine dinucleotide; GEO, Gene Expression Omnibus; HPA, Human Protein Atlas; siRNA, small interfering RNA; RT-q, reverse transcription-quantitative; CCK-8, Cell Counting Kit-8; SIRT, sirtuin

Key words: sirtuin 5, PI3K/AKT signaling pathway, GC-2 cell, asthenozoospermia

Introduction

As a prominent global health concern, infertility affects 8-12% of couples in the reproductive age group (between 15 and 49 years, with 50% being due to male-infertility-associated factors associated with abnormal semen parameters (1,2). Male infertility can be caused by congenital hypoplasia and varicocele (3). Its pathogenesis is usually caused by genetic and environmental factors (4). Studies have focused on key genes and signaling pathways that regulate male reproduction (5-8), but there is still controversy regarding this issue.

Sirtuins (SIRTs), highly conserved nicotinamide adenine dinucleotide (NAD)-dependent deacetylases, are involved in gene regulation, metabolism, aging and cancer (9). In mammals, seven types of SIRT, 1-7, have been identified with distinct structures, cellular localization and tissue expression (10). SIRTs are highly expressed in mammalian testicular tissue; to the best of our knowledge, few studies have evaluated the role of SIRTs in male reproductive function (11-13). SIRT2 has been implicated in regulating cell cycle progression and apoptosis in germ cells during spermatogenesis (14,15). Dysfunction of SIRT3 is associated with impaired sperm motility and increased sperm DNA damage, highlighting its role in maintaining sperm function (11,16,17). Loss of SIRT6 in mice results in an elevated number of apoptotic spermatids (18).

SIRT5 is a NAD-dependent desuccinylase, demalonylase and deacetylase protein (19). SIRT5 participates in a number of biological processes, including the urea cycle, fatty acid oxidation and amino acid metabolism (20). It also serves a key role in cellular antioxidant defense mechanisms by activating mitochondrial superoxide dismutase 2 (21,22). In addition, via regulation of DNA repair proteins, such as ATP-dependent DNA helicase 2 subunit Ku70-like protein, SIRT5 contributes to the maintenance of genomic integrity and protection against DNA damage-induced cellular dysfunction (23). The multifaceted functions of SIRT5 underscore its role in cell physiology, including maintaining metabolic homeostasis (22), regulating stress responses (24) and ensuring genomic stability (23). However, the specific functions and mechanisms of SIRT5 in spermatogenic cells remain to be explored.

The present study aimed to investigate the effects of SIRT5 on GC-2 spd cells and its underlying mechanisms to contribute to the development of more effective therapeutic strategies for asthenospermia.

Materials and methods

Human semen sample collection. The present human study was approved by the Ethics Committee of Shenzhen Ethics Review Committee on Biomedical Research (Shenzhen, China) [(2023) approval no. 001]. All participants provided written informed consent to participate. The subjects were patients who were treated in Peking University Shenzhen Hospital from July 2023 to January 2024. The present study included 25 male patients aged between 22 and 45 years with asthenozoospermia (sperm concentration, ≥15 million cells/ml; progressive motility, <32%; total motility, <40%; morphologically normal forms, ≥4%) and 25 participants with normal semen parameters. Semen samples were collected by masturbation following 3-7 days of abstinence. Samples were evaluated by computer-assisted semen analysis (version SSA-II, Suijia Software Co., Ltd) system. Inclusion criteria were as follows: i) Patients aged between 20 and 45 years with complete clinical data and ii) no syphilis, hepatitis, acquired immune deficiency syndrome or other infectious disease. Exclusion criteria were as follows: i) Patients with genitourinary tract infection; ii) patients with a history of recent use of drugs that interfere with sperm or semen quality and iii) malignant tumor or abnormal liver and kidney function.

Database mining. To compare the expression of SIRT5 among asthenozoospermic, asthenoteratozoospermic and normal sperm from fertile individuals, mRNA expression data was retrieved from the GEO database (accession no. GSE160749) (ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE160749). In addition, the protein expression of SIRT5 in testicular cells was queried using the Human Protein Atlas (HPA) database (proteinatlas.org/ENSG00000124523-SIRT5/single+cell+type).

Animals. A single male C57BL/6 J wildtype mouse (age, 2 months; weight, 22 g) was obtained from the Nanjing University Experimental Animal Institute (Nanjing, China). The mouse was housed in a specific-pathogen-free animal facility (22˚C, 55% humidity) with a 12/12-h light/dark cycle and free access to standard food and water. Health and behavior were monitored every day. The duration of the experiment was 20 weeks. Humane endpoints were as follows: Complete anorexia or signs of depression accompanied by hypothermia (body temperature <37˚C) without anesthesia or sedation. The mouse was euthanized by intraperitoneal injection of an overdose of 1% pentobarbital sodium (150 mg/kg). Animal death was confirmed by respiratory and cardiac arrest and pupil dilation was observed for ≥10 min. All animals were treated according to the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources for the National Research Council. The present study was approved by the Ethics Committee of Shenzhen Peking University-Hong Kong University of Science and Technology Medical Center (approval no. 2021-007).

Immunofluorescence staining. After the mouse was euthanized, the testicles were dissected and removed. Testes were fixed with 4% paraformaldehyde for 15-20 h at room temperature and dehydrated using a fully automatic tissue dehydrator (Excelsior AS; Thermo Fisher Scientific, Inc.). The samples were embedded in paraffin using an embedding machine (HistoStar; Thermo Fisher Scientific, Inc.), and sectioned to 3-5 μm thickness. Sections were deparaffinized in xylene at room temperature, rehydrated using descending ethanol concentrations, immersed in 0.01 M citrate buffer (Wuhan Servicebio Technology Co., Ltd.) at 100˚C for 10 min to facilitate antigen retrieval, and cooled for 2-3 h at room temperature. Sections were washed three times for 5 min each at room temperature with PBS (Gibco; Thermo Fisher Scientific, Inc.). Sections were blocked with 5% bovine serum albumin (BSA; Merck KGaA) at room temperature for 1h. Sections were incubated with SIRT5 (1:100; Cat. No. 15122-1-AP, Proteintech Group, Inc.) and γH2AX (1:100; ab26350, Abcam) in a humid environment at 4˚C overnight. Sections were washed in PBS and incubated with Alexa Fluor secondary antibody (1:100; Invitrogen; cat. # A-11008 and Cat. # A32742, Thermo Fisher Scientific, Inc.) for 1 h at room temperature and treated with VECTASHIELD® antifading mounting agent containing DAPI (Vector Laboratories, Inc.) for 10 min at room temperature. Sections were observed under a confocal microscope (STELLARIS 5; Leica GmbH).

To observe the mitochondrial staining, the GC-2 spd cells were cultivated in glass coverslips for 24 h and stained with Mito-Tracker red (Thermo Fisher Scientific, Inc.) for 30 min, all at 37˚C. Cells were blocked in 5% BSA for 1 h and incubated with SIRT5 (1:100) for 1 h at room temperature and sealed with DAPI, then observed under the confocal laser scanning microscope.

Cell culture. GC-2 spd cell line was procured from the American Type Culture Collection. Cells were maintained in a 25-cm^2 petri dish with DMEM containing 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin-streptomycin. Cells were maintained at 37˚C with 5% CO_2.

Plasmid transfection. GC-2 spd cells at 60-70%. GC-2 spd cells were transfected with 3 μg/ml pCDNA3.1-SIRT5 plasmid (Sangon Biotech Co. Ltd.) using Lipofectamine™ 3000 Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated in 5% CO_2 at 37˚C for 8 h to overexpress (OE) SIRT5. Cells transfected with 3 μg/ml pCDNA3.1 plasmid (Sangon Biotech Co. Ltd.) were used as a control. Cells were treated in a 5% CO_2 incubator at 37˚C for 48 h before subsequent experiments.

Small interfering (si)RNA transfection. SIRT5 and negative control siRNAs were synthesized by Suzhou GenePharma. The antisense sequences (5'→3') were as follows: Negative control, ACGUGACACGUUCGGAGAATT; SIRT5 siRNA-1, CCAGUUGUGUUGUAGACGATT and SIRT5 siRNA-2, GGCUCGUCCAAGUUCAAAUTT. When the cell density reached ~30% confluency, 1 μg/ml SIRT5 and control siRNAs were transfected into GC-2 spd cells. The cells were incubated at 37˚C in 5% CO_2 for 48 h before subsequent experiments.

Table 1. Primer sequences used in reverse transcription-quantitative PCR.

Target	Forward, 5'→3'	Reverse, 5'→3'
SIRT5 (human)	GGCACTTCCTCTGTGGTG	CGTAGCTGGGGTGGTCT
SIRT5 (mouse)	AAGCACATAGCCATCATCTC	CCCTCCGGTAGTGGTAAA
GAPDH (human)	CCACTCCTCCACCTTTGACG	CTGGTGGTCCAGGGGTCTTA
GAPDH (mouse)	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA

SIRT, sirtuin.

Table II. Basic semen parameters in patients with asthenozoospermia and healthy controls.

Variable	Control	Asthenozoospermia	P-value
Age, years	31.44±3.98	32.60±3.42	0.4594
Sperm count, x10^6 sperm/ml	94.72±21.05	80.20±40.31	0.1169
Volume, ml	4.28±1.17	4.30±1.07	0.9499
Total motility, %	77.24±5.72	26.40±9.05	<0.0001
Progressive motility, %	66.72±7.04	13.44±6.16	<0.0001

Data are presented as the mean ± SD.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from sperm samples and 1x10^6 GC-2 spd cells using SteadyPure RNA extraction kit (Hunan Accurate Bio-Medical Technology Co., Ltd.). According to the manufacturer's protocol, the extracted RNA was reverse-transcribed into cDNA using Evo M-MLV RT Mix Tracking kit with gDNA Clean for qPCR (Hunan Accurate Bio-Medical Technology Co., Ltd.). qPCR was carried out using SYBR Green Premix Pro Taq HS qPCR kit (Hunan Accurate Bio-Medical Technology Co., Ltd.). The following thermocycling conditions were used: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The housekeeping gene GAPDH was used as an internal control for normalization. RT-qPCR was performed out using a two-step process. The $2^{-\Delta\Delta Cq}$ method was employed to evaluate the relative expression of the target genes (25). The primers are listed in Table I.

Western blotting. Western blotting was performed as previously described (17). The antibodies were as follows: Anti-β-actin (1:10,000; cat. No. 66009-1-IG, Proteintech Group, Inc.), anti-SIRT5 (1:5,000; Cat. No. 15122-1-AP,Proteintech Group, Inc.), anti-PI3K (1:1,000; Cat. # 13666, Cell Signaling Technology, Inc.), anti-AKT (1:1,000; Cat. no. 9272, Cell Signaling Technology, Inc.,), anti-phosphorylated (p-)AKT (1:1,000; Cat. # 4060, Cell Signaling Technology, Inc.), anti-Bax (1:1,000; Santa Cruz Biotechnology, Inc.) and anti-Bcl-2 (1:1,000; Santa Cruz Biotechnology, Inc.). The secondary antibodies were anti-rabbit (1:2,000; Cat. #7074, Cell Signaling Technology, Inc.). and anti-mouse IgG (1:2,000; Cat. #7076, Cell Signaling Technology, Inc.).

Cell Counting Kit-8 (CCK-8). CCK-8 assay (Dojindo Molecular Technologies Inc.) was performed to evaluate cellular viability. Transfected GC-2 spd cells were seeded into a 96-well plate at a density of 3,000 cells/well. A volume of 10 μl CCK-8 (Beyotime Institute of Biotechnology) was added to the DMEM and incubated at 37˚C for 2 h. Subsequently, the optical density at 450 nm was measured every 24 h using a Multiskan Go plate reader (Beckman Coulter, Inc.).

EdU assay. An EdU incorporation assay was carried out with the EdU cell proliferation kit (cat. no. C0078L, Beyotime Institute of Biotechnology). Sterilized slides were placed in a 24-well plate and transfected cells were seeded into each well at a density of 30%. After culturing with 50 μmol/l EdU reagent for 2 h at room temperature, slides were washed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature (Beyotime Institute of Biotechnology). After staining the nucleus with DAPI, the cells were imaged and photographed with a confocal laser scanning microscope.

Wound healing assay. Cell migration was detected using a wound healing assay. 5x10^5 transfected cells were plated in a 6-well plate and cultured to 90% confluency. Subsequently, monolayers were scratched with a 200 μl pipette tip and washed with PBS. The medium was replaced with a serum-free DMEM and images were captured 0 and 24 h by a Lecia DMi8 fluorescence microscope. All cells were grown at 37˚C in a 5% CO_2 incubator.

Apoptosis assay. An apoptosis assay was performed using Annexin V, 633 Apoptosis Detection kit (Dojindo Laboratories, Inc.). In brief, after digestion with trypsin, the cells were centrifuged at 700 x g for 2 min at 4˚C, washed twice with PBS and resuspended in binding buffer at a final density of 1x10^6 cells/ml. Annexin V-633 and PI (5 μl each) were added

to 100 μl cell suspension. The cell suspension was mixed and incubated for 15 min at room temperature in the dark. A total of 200 μl binding buffer was added and cells were measured by flow cytometry using Calibur (BD Accuri C6 Plus; BD Biosciences). Data were analyzed using BD Accuri C6 Plus software (version 1.0.264.21; BD Biosciences) and the apoptotic rate was calculated as the percentage of early (Annexin V-FITC) + late apoptotic (Annexin V-FITC and PI) cells.

Co-immunoprecipitation. A total of $1x10^7$ GC-2 cells were lysed in 1 ml NP-40 buffer (Beyotime Institute of Biotechnology) containing protease inhibitor cocktail, centrifuged at 12,000 g for 10 min at 4˚C, and the supernatant was removed for use. 1 ml lysates were incubated with PI3K (1:100; Cat. # 13666, Cell Signaling Technology, Inc.), FLAG (1:200; Cat. No. 20543-1-AP, Wuhan Sanying Biotechnology) or mouse IgG (1:100; sc-515946, Santa Cruz Biotechnology, Inc.) antibody for 12 h at 4˚C and incubated with 20 μl Pierce Protein A/G Magnetic Beads (Thermo Fisher Scientific, Inc.) for 1 h at room temperature. The beads were washed with PBST (0.1% Tween-20) and incubated at 95˚C for 10 min in 1x loading buffer (Beyotime Institute of Biotechnology). The beads were separated using a magnetic rack and the supernatant was used for Western blotting, as aforementioned.

Statistical analysis. Data analysis was performed using GraphPad Prism 8 (Dotmatics). The results from three experiments are reported as mean ± standard deviation. Student's unpaired t-test was employed for comparisons between two independent groups, while one-way ANOVA followed by the Tukey's post hoc test was used to assess significance between >2 groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

SIRT5 is downregulated in asthenozoospermia and asthenoteratozoospermia. In our previous study, proteomic analysis revealed that SIRT5 was downregulated in asthenozoospermia compared with normal sperm samples (Fig. 1A) (26). In addition, the expression levels of SIRT5 were assessed in asthenozoospermia, asthenoteratozoospermia and normal sperm from fertile men by downloading the data from the GEO database (GSE160749). SIRT5 expression was significantly downregulated in asthenozoospermia and asthenoteratozoospermia spermatozoa compared with normal (Fig. 1B). We obtained samples from clinical patients and the basic semen parameters in normal and asthenozoospermic patients are described in Table II. Furthermore, expression of SIRT5 was assessed in normal and asthenozoospermia sperm samples, which revealed that its mRNA and protein expression was significantly downregulated in asthenozoospermia sperm samples (Fig. 1C and D).

SIRT5 is expressed in the testis and primarily expressed in mitochondria in spermatocytes. To determine the SIRT5 expression in the testicular tissue, immunofluorescence analysis was conducted using testicular tissue from a 2-month-old wild-type mouse. Analysis revealed widespread expression of SIRT5 in testicular tissue, including its presence in spermatocytes (Fig. 1E). This was congruent with the expression profile of SIRT5 in the HPA database (Fig. 1G). In the mouse spermatocyte cell line GC-2 spd, SIRT5 was mainly localized to mitochondria (Fig. 1F).

SIRT5 promotes the proliferation of GC-2 spd cells. Both gain- and loss-of-function assays were performed to further elucidate the biological function of SIRT5 in GC-2 spd cells. GC-2 spd cells were infected with lentivirus harboring pLV-SIRT5 or siRNAs for SIRT5 knockdown. The efficacy of SIRT5 modulation in GC-2 spd cells was evaluated by RT-qPCR and western blot analysis (Fig. 2A-D). CCK-8 assays revealed the viability of OE-SIRT5 GC-2 spd cells increased compared with the vector control group (Fig. 2E). Conversely, knockdown of SIRT5 in GC-2 spd cells decreased viability (Fig. 2E). EdU assay revealed that SIRT5 knockdown inhibited cell proliferation while OE-SIRT5 increased cell proliferation (Fig. 2F). These results suggested that SIRT5 promoted the proliferation of GC-2 spd cells.

SIRT5 facilitates the migration of GC-2 spd cells. Wound healing assay was performed to investigate the effects of SIRT5 on the motility of GC-2 spd cells. Knockdown of SIRT5 significantly impaired the migratory capacities of GC-2 spd cells (Fig. 3A), whereas OE increased migration (Fig. 3B).

SIRT5 inhibits apoptosis of GC-2 spd cells. To determine the effects of SIRT5 on apoptosis in GC-2 spd cells, flow cytometry was used. Notably, the proportion of apoptotic cells was significantly elevated in the SIRT5 knockdown compared with the control group (Fig. 4A). Conversely, the proportion of apoptotic cells with OE-SIRT5 significantly decreased (Fig. 4B).

SIRT5 promotes the proliferation of GC-2 spd cells via the PI3K/AKT pathway. To clarify the molecular mechanism underlying regulation of proliferation and apoptosis by SIRT5 in GC-2 spd cells, western blotting was performed. Knockdown of SIRT5 suppressed the expression of Bcl-2 and p-AKT, but promoted the expression of Bax (Fig. 5A and B). Conversely, OE-SIRT5 markedly enhanced the expression of Bcl-2 and p-AKT, while decreasing expression of Bax (Fig. 5A and B). To explore how SIRT5 regulates the PI3K/AKT pathway, the binding of SIRT5 to PI3K was assayed using co-immunoprecipitation (Fig. 5C). SIRT5 could bind with PI3K, which might inhibit the phosphorylation of PI3K/AKT signaling members.

Discussion

The causes of male infertility are complicated, and its pathogenesis is unclear (27). The process of spermatogenesis is regulated by factors including energy metabolism, signaling pathways, and REDOX processes, to ensure normal viability and fertilization ability (28,29). Our previous proteomic analysis revealed that SIRT5 is significantly downregulated in asthenozoospermic sperm (26); this finding was validated in human sperm samples. However, the specific function of SIRT5 in male germ cells has not been validated. To the best of our knowledge, the present study is the first to demonstrate the role of SIRT5 in inhibiting apoptosis and promoting sperm cell proliferation and migration *in vitro*. The present study

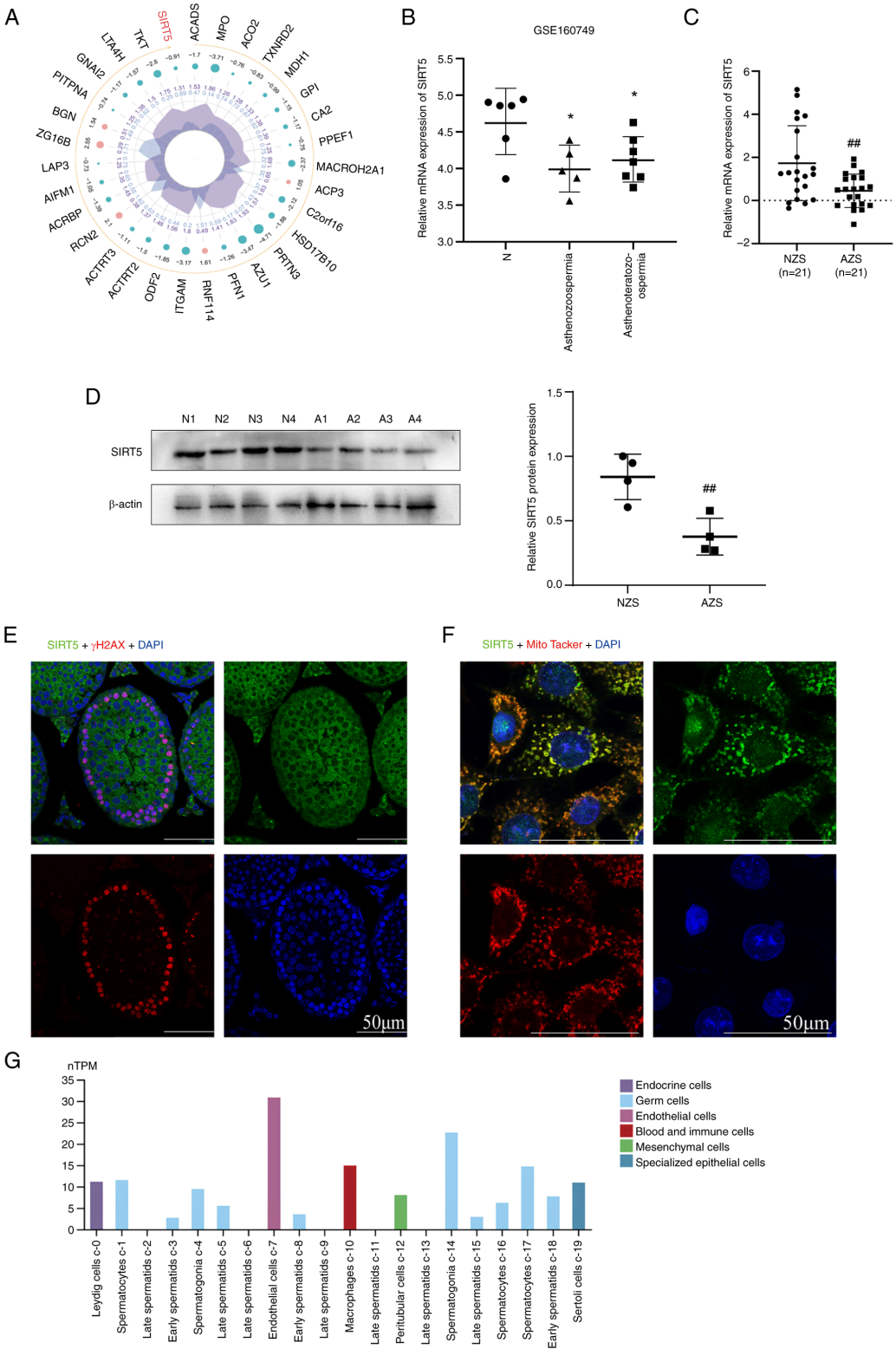

Figure 1. Expression of SIRT5 in human and mouse sperm. (A) Previous proteomics analysis revealed that SIRT5 expression was significantly decreased in asthenozoospermia compared with normal sperm (26). (B) Analysis of the GSE160749 data set revealed that the mRNA expression of SIRT5 in asthenozoospermia and asthenoteratozoospermia sperm was decreased compared with normal sperm. (C) Expression of SIRT5 in asthenozoospermia sperm samples was lower compared with that in 21 normal samples. (D) SIRT5 protein expression between normal and asthenozoospermia sperm samples. Immunofluorescent co-localization of SIRT5 and (E) marker of spermatocytes in meiosis (γH2AX) in murine testes and (F) mitochondria. Scale bar, 100 μm. (G) SIRT5 expression in testicular cell species. *P<0.05 vs. N; $^{\#\#}$P<0.01 vs. NZS. SIRT5, sirtuin 5; N, normal; A, asthenozoospermia; NZS, normozoospermic; AZS, asthenozoospermic; TPM, transcripts per million.

revealed that SIRT5 was involved in proliferation of GC-2 spd cells by regulating the PI3K/AKT pathway.

Both the aforementioned proteomics results and analysis of data from the GEO database revealed that SIRT5 was

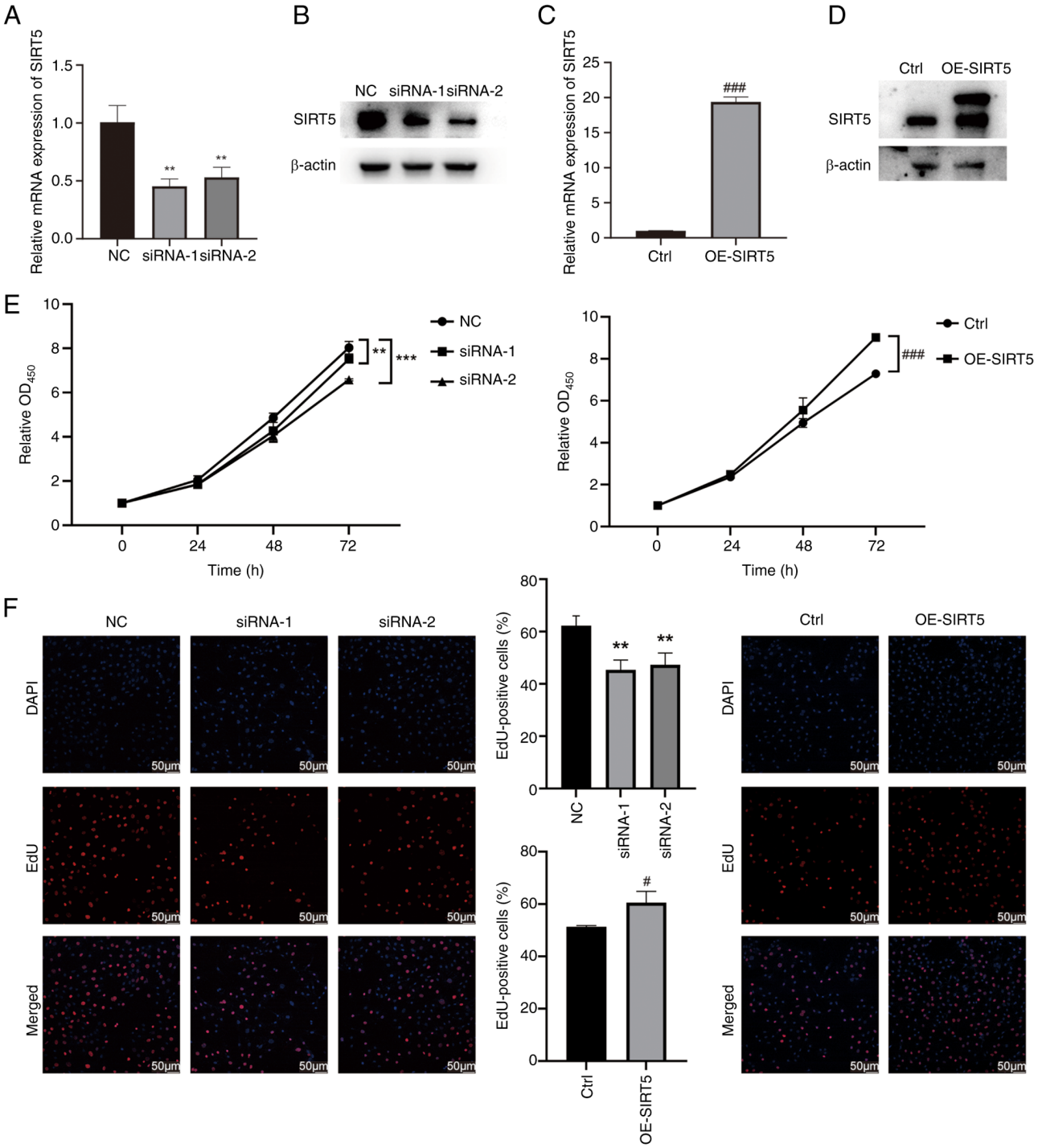

Figure 2. Knockdown of SIRT5 inhibits proliferation of GC-2 spd cells. Following transfection with siRNAs, SIRT5 expression was suppressed in GC-2 spd cells at both (A) transcriptional and (B) translational levels. After infection with OE-SIRT5 lentivirus, SIRT5 expression was markedly increased in GC-2 spd cells at both (C) transcriptional and (D) translational levels. (E) Viability of GC-2 spd cells treated with siRNAs or lentivirus. (F) EdU assays revealed the proliferation capacity of GC-2 spd cells treated with siRNAs or lentivirus. **P<0.01, ***P<0.001 vs. NC; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 vs. Ctrl. Ctrl, control; si, small interfering; NC, negative control; OD, optical density; SIRT5, sirtuin 5; OE, overexpression.

significantly downregulated in asthenozoospermia compared with normal motile sperm, which was also confirmed by RT-qPCR in human sperm samples. These findings aligned with those reported in previous studies (30,31). SIRT5 was expressed in the testis and mainly localized in the mitochondria in the mouse spermatogenic cell line. Loss of SIRT5 leads to mitochondrial membrane potential defects and oxidative stress damage (32-34), which can interfere with sperm motility (35,36). Therefore, it was hypothesized that SIRT5 has an important role in maintaining the normal movement of mature sperm.

The present study revealed that knockdown of SIRT5 decreased viability and proliferation and increased apoptosis of GC-2 spd mouse spermatocytes. Previous research has reported that SIRT5 participates in cellular processes through its regulation of the PI3K/AKT pathway (37). This pathway

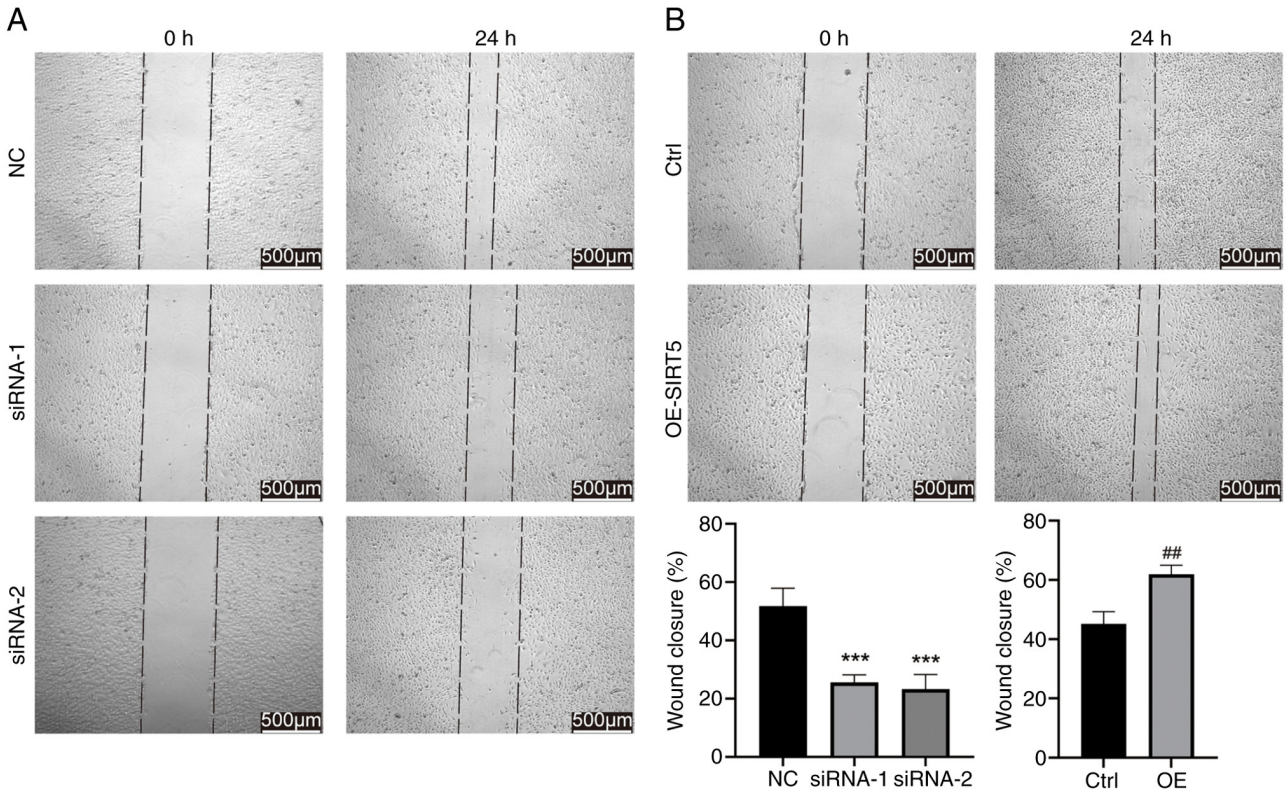

Figure 3. SIRT5 facilitates migration in GC-2 spd cells. (A) Wound healing assay of GC-2 spd cells transfected with SIRT5 (A) siRNA and (B) OE lentivirus. ***P<0.001 vs. NC; ##P<0.01 vs. Ctrl. SIRT5, sirtuin 5; Ctrl, control; NC, negative control; siRNA, small interfering RNA; OE, overexpression.

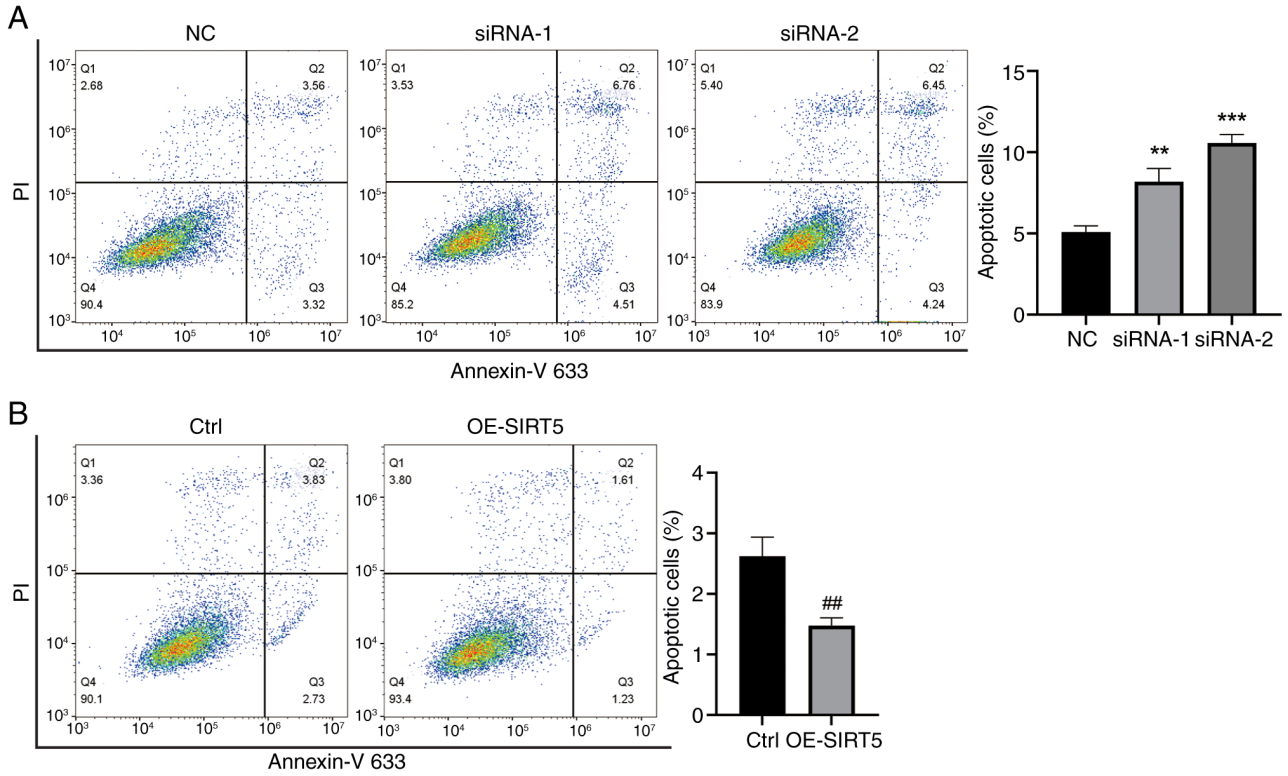

Figure 4. SIRT5 inhibits apoptosis in GC-2 spd cells. (A) Proportion of apoptotic SIRT5 (A) knockdown and (B) OE cells. **P<0.01, ***P<0.001 vs. NC; ##P<0.01 vs. Ctrl. SIRT5, sirtuin 5; Ctrl, control; NC, negative control; siRNA, small interfering RNA; OE, overexpression.

serves a key role in governing spermatogonial stem cell self-renewal and spermatogonial proliferation (38). Therefore, we explored the relationship between SIRT5 and the PI3K/AKT pathway and found that the interaction between SIRT5 and

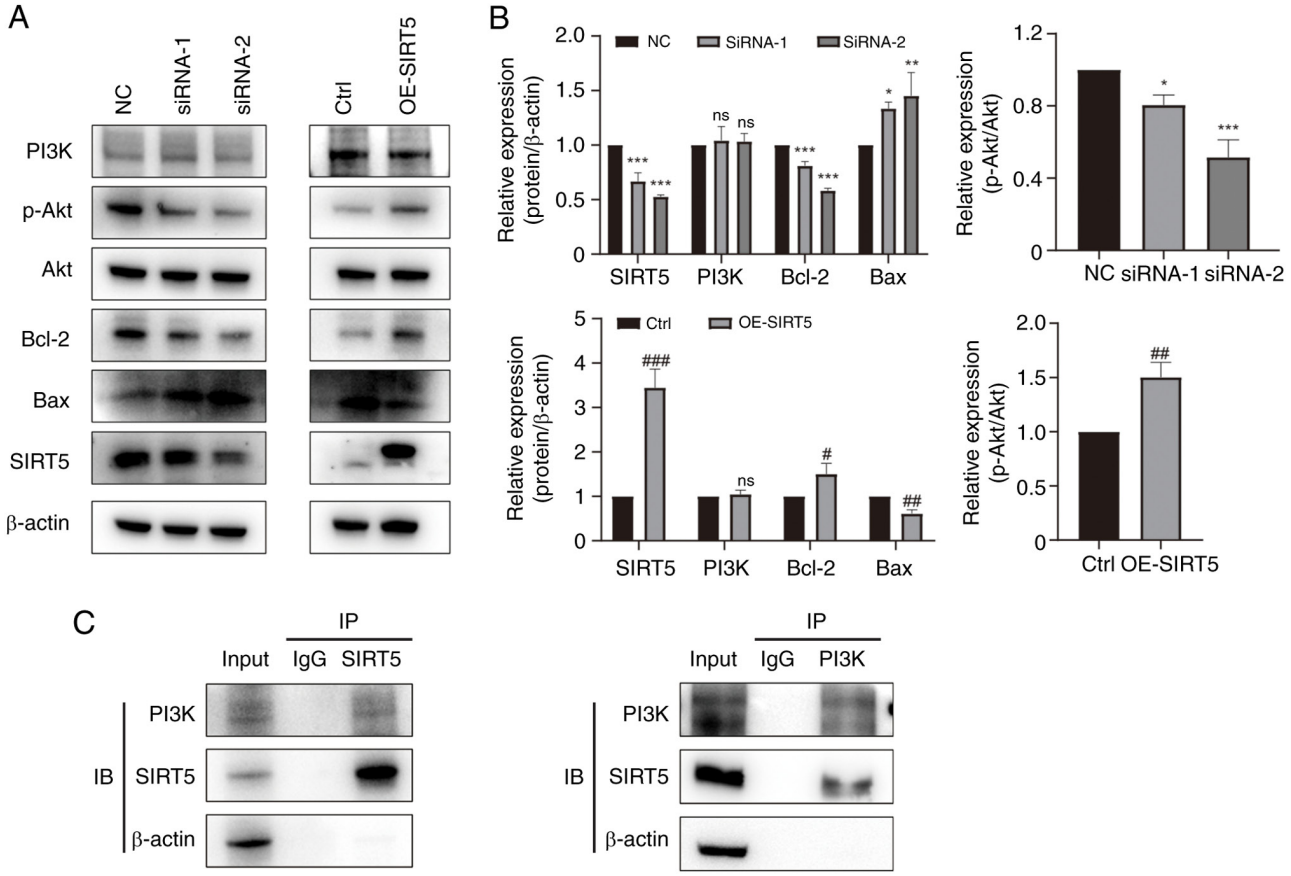

Figure 5. SIRT5 regulates the PI3K/AKT signaling pathway in GC-2 spd cells. (A) Western blot analysis of PI3K, p-AKT, AKT, Bcl-2, Bax and SIRT5 in GC-2 spd cells treated with siRNA or lentivirus. (B) Semi-quantitative analysis of western blot results. (C) Co-IP using a PI3K and FLAG antibody on GC-2 spd cells. *P<0.05, **P<0.01, ***P<0.001 vs. NC; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. Ctrl. p-, phosphorylated; SIRT5, sirtuin 5; Ctrl, control; NC, negative control; ns, not significant; siRNA, small interfering RNA; OE, overexpression; IB, immunoblot; IP, immunoprecipitation.

PI3K was responsible for inhibiting the phosphorylation of the PI3K/AKT signaling pathway members. Studies have revealed that the PI3K/AKT signaling pathway inhibits sperm apoptosis, accompanied by a decrease in the expression of Bax (39,40), which is consistent with the findings of the present study. In addition, inhibiting the PI3K/AKT signaling pathway decreases the expression of premeiotic and meiotic markers in the testes of postnatal and adult mice (41), whereas activation of PI3K/AKT signaling promotes meiotic entry (42). Meiosis is a complex process that begins in the primary spermatocyte and requires several proteins and enzymes (43). Abnormal meiosis has a marked effect on both sperm count and quality, increasing the risk of infertility (44,45). Therefore, it was hypothesized that SIRT5 may be an important gene affecting male infertility via the regulation of PI3K/AKT pathway.

SIRT5 has low deacetylation activity but strong desuccinylation, demalonylation and deglutarylation activities (18,19). For example, SIRT5 regulates the malonylation of GAPDH and succinylation of pyruvate kinase M2 (46,47) and isocitrate dehydrogenase 2 (48) to alter their activity, thereby participating in the regulation of glycolysis and the tricarboxylic acid cycle. It was hypothesized that altered SIRT5 expression may cause disturbances in energy metabolism and homeostasis via these metabolic changes, which in turn would affect germ cell proliferation and apoptosis. Especially in mature sperm, ATP is primarily produced through glycolysis to maintain sperm motility. Therefore, it was hypothesized that changes in SIRT5 expression in sperm would exert an effect on sperm motility by influencing the glycolytic pathway (49). In addition, SIRT5 interacts with PI3K, therefore, SIRT5 may affect the PI3K/AKT pathway through the regulation of the protein modifications. However, further studies are needed to clarify the role of SIRT5 post-translational modification in regulation of spermatogenesis and sperm viability.

The present study revealed that SIRT5 is associated with sperm motility, and served an important role in maintaining the viability and proliferation of GC-2 cells and decreasing apoptosis. The results of the present study are important for a comprehensive understanding of the mechanism and treatment of male infertility. However, the lack of *in vivo* data to assess how SIRT5 affects infertility warrants further investigation.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HX performed experiments and data analysis. HX, SC and MW constructed figures, data interpretation, and wrote the manuscript. HX, TZ and BY designed, supervised the study, and confirmed the authenticity of all the raw data. TZ and BY provided financial support. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All experiments strictly followed the 'Helsinki Declaration' and obtained ethical approval from the Ethics Committee of Shenzhen Ethics Review Committee on Biomedical Research (Shenzhen, China) [approval no. (2023) 001]. All patients signed written informed consent. All animal protocols were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee of Shenzhen Peking University-Hong Kong University of Science and Technology Medical Center (Shenzhen, China) (approval no. 2021-007).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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