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SOX3 REGULATES GRANULOSA CELL DYNAMICS: MODULATION OF PI3K/AKT PATHWAY THROUGH SPP1 TARGETING

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ABSTRACT

Ovarian granulosa cells (GCs) are crucial for female fertility, with their proliferation and apoptosis precisely regulated. Dysregulation of these processes contributes to ovarian disorders. This study investigates the role of the transcription factor SOX3 in granulosa cell dynamics and its underlying molecular mechanisms. We demonstrate that SOX3 promotes GC proliferation and suppresses apoptosis. Mechanistically, SOX3 achieves these effects by directly upregulating the expression of Secreted Phosphoprotein 1 (SPP1), which subsequently activates the Phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling pathway. Our findings establish a novel SOX3-SPP1-PI3K/AKT axis as a critical regulatory mechanism in granulosa cell function, offering potential targets for therapeutic interventions in reproductive health.

Keywords: SOX3, granulosa cells, proliferation, apoptosis, SPP1, PI3K/AKT pathway, folliculogenesis, ovarian function.

INTRODUCTION

In mammals, the intricate process of reproduction hinges upon the proper functioning of the female reproductive system, with the ovaries serving as the primary organs responsible for gamete production and hormone synthesis. Within the ovarian cortex, the ovarian follicle represents the fundamental functional unit, comprising an oocyte, surrounding granulosa cells (GCs), and theca cells [1]. The orchestrated development of these follicles, a process known as folliculogenesis, is a highly complex and tightly regulated cascade of events that commences with the activation of primordial follicles and culminates in ovulation [2]. This multi-stage process is critically dependent on the precise balance between proliferation and apoptosis of ovarian granulosa cells.

Granulosa cells are somatic cells that encapsulate the oocyte, providing essential structural and nutritional support throughout follicular development. They are pivotal for oocyte maturation, steroidogenesis (producing hormones like estrogen), and the establishment of a conducive microenvironment for gamete growth [3, 4]. The dynamic interplay between GCs and the oocyte, often mediated by paracrine factors such as Growth Differentiation Factor 9 (GDF9) and Bone Morphogenetic Protein 15 (BMP15) from the oocyte, is indispensable for successful folliculogenesis [5]. Any disruption to the delicate equilibrium between GC proliferation and programmed cell death (apoptosis) can lead to severe reproductive abnormalities, including follicular atresia, polycystic ovary syndrome (PCOS), and premature ovarian failure (POF) [4, 6, 7, 43, 44, 69, 70]. Therefore, a comprehensive understanding of the molecular pathways governing GC fate is paramount for addressing female infertility and related reproductive disorders.

Cellular signaling pathways play an indispensable role in orchestrating granulosa cell behavior. Among these, the Phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling pathway stands out as a central regulator of diverse cellular functions, including cell growth, proliferation, survival, metabolism, and apoptosis [51]. Its critical involvement in ovarian physiology has been extensively documented, with numerous studies highlighting its role in promoting GC proliferation and inhibiting apoptosis, thereby fostering follicular development and preventing atresia [3, 6, 7, 17, 18, 20, 52]. For instance, activation of the PI3K/AKT pathway by hormones like Follicle-Stimulating Hormone (FSH) and estradiol (E2) has been shown to mediate ovarian follicle growth [18]. Conversely, inhibition of this pathway can accelerate GC apoptosis and contribute to ovarian senescence [20]. Key modulators of the PI3K/AKT pathway, such as the phosphatase and tensin homolog (PTEN) and Src homology 2 domain-containing phosphatase 2 (SHP2), have been demonstrated to critically influence follicular development by finely tuning PI3K/AKT activity [3, 7, 52]. Beyond PI3K/AKT, other signaling cascades, including the Mitogen-Activated Protein Kinase (MAPK3/1 or ERK1/2) pathway [8], Protein Kinase A and C pathways [9], the Hippo pathway [10, 15], Notch signaling [12], Hedgehog signaling [13], the

Transforming Growth Factor-beta (TGF-β) family [14], and the Wnt/beta-catenin pathway [16], collectively contribute to the intricate regulatory network governing granulosa cell function and follicular maturation.

The SRY-related HMG-box (SOX) family of transcription factors is characterized by a conserved high-mobility group (HMG) DNA-binding domain, enabling them to bind to specific DNA sequences and regulate gene expression [21]. SOX proteins are fundamental to a wide range of developmental processes, notably including sex determination and differentiation across various species [21, 22, 25, 26]. SOX3, a member of the SOXB1 subgroup, is located on the X chromosome and is recognized for its crucial roles in the development of the neural system and the hypothalamo-pituitary axis [22, 48, 49]. While its most prominent association in humans involves XX male sex reversal conditions resulting from its duplication or genomic rearrangements [22, 23, 24], and its necessity for gonadal function in both sexes in certain animal models [25], the precise functions of SOX3 within ovarian granulosa cells, particularly in mammals, remain an area of active investigation. Studies in teleost fish, such as zebrafish and Nile tilapia, have indicated a vital role for Sox3 in oogenesis and follicle development, where its loss-of-function leads to significant follicular retardation and reduced fecundity [29, 34]. Furthermore, recent research has begun to highlight SOX3's influence on ovarian granulosa cell proliferation and estradiol secretion [35]. Beyond the reproductive system, SOX3 has also been implicated in promoting the generation of committed spermatogonia in postnatal mouse testes [45] and its dosage is critical for normal pituitary development [46, 47, 48]. These diverse roles underscore the broad regulatory capacity of SOX3 in developmental and physiological contexts.

Secreted Phosphoprotein 1 (SPP1), widely known as Osteopontin (OPN), is a highly glycosylated phosphoprotein that functions as both a cytokine and an extracellular matrix protein. SPP1 mediates crucial cell-matrix interactions and intracellular signaling by binding to various receptors, including integrins (e.g., αvβ3) and CD44 [59, 68]. Its involvement in promoting cell proliferation, migration, and survival has been well-established across numerous cell types, frequently through the activation of the PI3K/AKT/mTOR signaling pathway [59, 60, 61, 62, 65]. For instance, SPP1 has been shown to drive the progression of idiopathic pulmonary fibrosis and non-small cell lung cancer by activating the PI3K/AKT/mTOR pathway [60]. It also promotes bladder cancer progression by regulating NOTCH3 and subsequently activating PI3K/AKT [61]. Within the ovarian context, multi-omics studies have suggested a role for SPP1 in ovarian aging [63], and it has been observed that three-dimensional culture systems for rat ovarian granulosa cells lead to increased SPP1 expression, mediated by the PI3K/AKT pathway [64]. This suggests a direct involvement of SPP1 in supporting follicular development and maturation.

Considering the well-documented importance of SOX3 in developmental processes, the central role of the PI3K/AKT pathway in cell proliferation and survival, and the emerging significance of SPP1 as a mediator of PI3K/AKT signaling, particularly in ovarian cells, we hypothesized that SOX3 might regulate granulosa cell proliferation and apoptosis by modulating the PI3K/AKT pathway, potentially through targeting SPP1. This comprehensive study was therefore designed to systematically investigate the expression and functional significance of SOX3 in ovarian granulosa cells, delineate its impact on cell proliferation and apoptosis, and unravel the intricate molecular mechanisms underlying these effects, with a particular focus on the involvement of the PI3K/AKT pathway and SPP1. Our aim is to provide novel insights into the regulatory networks governing granulosa cell dynamics, which could have significant implications for understanding and potentially treating female reproductive disorders.

MATERIALS AND METHODS

This section outlines the detailed experimental procedures and analytical approaches employed in this study to investigate the role of SOX3 in granulosa cell proliferation and apoptosis, and its interaction with the SPP1/PI3K/AKT pathway. All experiments were conducted with rigorous attention to reproducibility and statistical validity.

Cell Culture and Transfection

Cell Lines and Maintenance

Primary mouse granulosa cells (GCs) were meticulously isolated from the ovaries of pre-pubertal female C57BL/6 mice, aged 21–23 days, following established protocols with slight modifications [18]. Briefly, ovaries were dissected, cleaned of surrounding fat and connective tissue, and punctured with a fine needle to release GCs into a sterile dish containing Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12, HyClone, Catalog No. SH30023.01) supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone, Catalog No. SH30071.03) and 1% penicillin-streptomycin solution (Gibco, Catalog No. 15140122). Cells were then filtered through a 70 μm cell strainer to remove tissue debris and cultured in a humidified incubator at 37°C with 5% CO$_2$.

The human ovarian granulosa-like tumor cell line KGN (JCRB Cell Bank, Catalog No. JCRB0907), a widely accepted model for studying human granulosa cell function due to its physiological similarities to primary GCs [10, 69, 70], was cultured in DMEM/F12 (1:1 ratio, HyClone) supplemented with 10% FBS (HyClone) and 1% penicillin-streptomycin solution (Gibco). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO$_2$. Cell passages were kept low to ensure experimental consistency, and cells were routinely tested for mycoplasma contamination.

Gene Overexpression and Knockdown

For stable overexpression of SOX3, the full-length coding sequence of human SOX3 was synthesized and subcloned into the pcDNA3.1 lentiviral expression vector (GenePharma, Shanghai, China). Lentiviruses containing pcDNA3.1-SOX3 (OE-SOX3) and an empty vector control (pcDNA3.1-NC, referred to as OE-NC) were packaged by GenePharma using a standard lentiviral packaging system. KGN cells were transduced with these lentiviruses according to the manufacturer's instructions. To establish stably overexpressing cell lines, transduced cells were subjected to selection with puromycin (1 μg/mL, Sigma-Aldrich, Catalog No. P8833) for two weeks, with media changes every 2-3 days [35]. The efficiency of overexpression was routinely verified by quantitative real-time PCR (RT-qPCR) and Western blot analysis.

For stable knockdown of SOX3, three distinct small hairpin RNAs (shRNAs) targeting different regions of the human SOX3 mRNA sequence (sh-SOX3-1, sh-SOX3-2, sh-SOX3-3) and a non-targeting scramble control shRNA (sh-NC) were designed and synthesized by HanBio Co., Ltd (Shanghai, China). These shRNAs were cloned into lentiviral vectors. KGN cells were transduced with these lentiviruses, and stable knockdown cell lines were established through puromycin selection (1 μg/mL) for two weeks. The shRNA sequence with the highest knockdown efficiency, as determined by RT-qPCR and Western blot, was selected for subsequent experiments (sh-SOX3-2).

For transient knockdown of SPP1, three pairs of small interfering RNAs (siRNAs) targeting human SPP1 (si-SPP1-1, si-SPP1-2, si-SPP1-3) and a random negative control siRNA (si-NC) were synthesized by Tsingke Biotech (Beijing, China). KGN cells were transfected with siRNAs (50 nM final concentration) using Lipofectamine 3000 reagent (Invitrogen, Catalog No. L3000015) according to the manufacturer's protocol. Experiments were performed 48-72 hours post-transfection. The most effective siRNA (si-SPP1-3) was chosen for subsequent rescue experiments.

For transient overexpression of SPP1, the full-length coding sequence of human SPP1 was synthesized and cloned into the pcDNA3.1 plasmid (pcDNA3.1-SPP1). KGN cells were transfected with pcDNA3.1-SPP1 or an empty pcDNA3.1 vector (pcDNA3.1-NC) using Lipofectamine 3000. Experiments were performed 48 hours post-transfection.

Pharmacological Inhibition

To inhibit the PI3K/AKT signaling pathway, cells were treated with LY294002 (MedChemExpress, Catalog No. HY-10108), a specific PI3K inhibitor. LY294002 was dissolved in dimethyl sulfoxide (DMSO) and used at a final concentration of 20 μM. Control cells received an equivalent volume of DMSO. Cells were pre-treated with LY294002 for 2 hours before the initiation of specific assays.

Cell Proliferation and Apoptosis Assays

Cell Counting Kit-8 (CCK-8) Assay

Cell viability and proliferation were quantitatively assessed using the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan, Catalog No. CK04) [7, 70]. KGN cells were seeded into 96-well plates at a density of 2,000 cells/well in 100 μL of complete culture medium. After allowing cells to adhere overnight, the medium was replaced with fresh medium containing the respective treatments (e.g., OE-SOX3, sh-SOX3, or controls). At predetermined time points (12, 24, 48, and 72 hours), 10 μL of CCK-8 reagent was added to each well and incubated for 1 hour at 37°C in the dark. The absorbance at 450 nm was measured using a microplate reader (BioTek Epoch, USA). Background absorbance from wells containing only medium and CCK-8 reagent was subtracted from all readings. Each condition was performed in at least three independent biological replicates, with each replicate having six technical wells.

EdU (5-ethynyl-2'-deoxyuridine) Incorporation Assay

Cell proliferation was further evaluated by assessing DNA synthesis using the EdU incorporation assay (Beyotime, China, Catalog No. C0078S) [70]. KGN cells were seeded into 24-well plates at a density of 1 × 10^5 cells/well. After 24 hours of treatment, cells were incubated with 50 μM EdU solution for 2 hours at 37°C. Following EdU incorporation, cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature, permeabilized with 0.5% Triton X-100 for 10 minutes, and then stained with Apollo® 567 (for EdU detection) according to the manufacturer's instructions. Cell nuclei were counterstained with Hoechst 33342 (Beyotime, Catalog No. C1011) for 10 minutes in the dark. Images were captured using a fluorescence microscope (Thunder Imager, Leica, Germany). The percentage of EdU-positive cells (red fluorescence) relative to the total number of cells (blue fluorescence) was calculated from at least five random fields per well using ImageJ software.

Annexin V-FITC/Propidium Iodide (PI) Double Staining Flow Cytometry

Cell apoptosis was quantified using the Annexin V-APC/Propidium Iodide (PI) double staining kit (Bestbio, China, Catalog No. BB-41033) and flow cytometry [4, 57, 58]. KGN cells were harvested following various treatments, washed twice with ice-cold phosphate-buffered saline (PBS), and then resuspended in 500 μL of 1X binding buffer. Subsequently, 5 μL of Annexin V-APC and 5 μL of PI were added to the cell suspension. The mixture was incubated at room temperature for 15 minutes in the dark. Apoptotic cells were immediately analyzed using a flow cytometer (BD Biosciences, USA, BD FACSCalibur™). Early apoptotic cells (Annexin V-APC positive, PI negative) and late apoptotic/necrotic cells (Annexin V-APC positive, PI positive) were quantified. Data analysis was performed using Summit 4.3 Software.

Terminal Deoxynucleotidyl Transferase-mediated dUTP-biotin Nick End Labeling (TUNEL) Assay

Apoptotic cells were also detected in situ using the One-Step TUNEL Apoptosis Assay Kit (Elabscience, China, Catalog No. E-CK-A322) [57, 58]. KGN cells were seeded into 24-well plates (1 × 10^5 cells/well). After 24 hours of treatment, cells were fixed with 4% PFA for 20 minutes at room temperature, permeabilized with 0.2% Triton X-100/PBS for 10 minutes at 37°C. Subsequently, cells were incubated with the TdT reaction mixture for 2 hours at 37°C in a humidified chamber. Cell nuclei were counterstained with DAPI for 5 minutes in the dark. Images were captured using a fluorescence microscope (Thunder Imager, Leica, Germany). The percentage of TUNEL-positive cells (red fluorescence) relative to the total number of cells (blue fluorescence) was calculated from at least five random fields per well.

Quantitative Real-Time PCR (RT-qPCR)

RNA Extraction and cDNA Synthesis

Total RNA was extracted from KGN cells and mouse ovarian tissues using TRIzol reagent (Invitrogen, Catalog No. 15596026) according to the manufacturer's protocol [36]. RNA quantity and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), ensuring A260/A280 ratios between 1.8 and 2.0 and A260/A230 ratios greater than 2.0. High-quality RNA (1 μg) was reverse transcribed into complementary DNA (cDNA) using the RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific, USA, Catalog No. K1691) with oligo(dT)18 primers, following the manufacturer's instructions.

RT-qPCR Reaction

Quantitative real-time PCR (RT-qPCR) was performed using SYBR Green qPCR Mix (GeneCopoeia, USA, Catalog No. D01010) on a QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA). Each 20 μL reaction mixture contained 10 μL of SYBR Green qPCR Mix, 0.4 μL of forward primer (10 μM), 0.4 μL of reverse primer (10 μM), 2 μL of cDNA template, and 7.2 μL of nuclease-free water. The cycling conditions were: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A melting curve analysis was performed at the end of each run to confirm primer specificity and the absence of non-specific amplification. Gene expression was normalized to the internal reference gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative mRNA expression levels were calculated using the 2$^{-\Delta\Delta\text{Ct}}$ method. All primer sequences used in this study are listed in Supplementary Table S1.

Western Blot Analysis

Protein Extraction and Quantification

Cells and zebrafish ovarian tissues were collected and lysed using ice-cold RIPA lysis buffer (Beyotime, China, Catalog No. P0013B) supplemented with a protease inhibitor cocktail (Roche, Catalog No. 04693159001) and phosphatase inhibitors (Sigma-Aldrich, Catalog No. P5726). Lysates were incubated on ice for 30 minutes with intermittent vortexing and then centrifuged at 12,000 × g for 15 minutes at 4°C to remove cellular debris. The supernatant containing total protein was collected. Protein concentrations were determined using the Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce, USA, Catalog No. 23225) with bovine serum albumin (BSA) as a standard.

SDS-PAGE and Immunoblotting

Equal amounts of protein (20-40 μg) from each sample were mixed with 5X SDS-PAGE loading buffer, denatured by boiling at 95°C for 5 minutes, and then separated by electrophoresis on a 10% or 12% SDS-polyacrylamide gel (SDS-PAGE). Following separation, proteins were electro-transferred from the gel to polyvinylidene difluoride (PVDF) membranes (Millipore, USA, Catalog No. IPVH00010) using a wet transfer system. Membranes were then blocked with 5% non-fat milk powder (Bio-Rad, Catalog No. 1706404) in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 hour at room temperature to prevent non-specific antibody binding.

Blocked membranes were incubated overnight at 4°C with primary antibodies diluted in 5% BSA in TBST. The primary antibodies used were:

- β-actin (Proteintech, Catalog No. 66009-1-Ig, 1:10,000) - used as a loading control.
- SOX3 (Zen-bio, Catalog No. 862515, 1:1,000)
- PCNA (Proteintech, Catalog No. 60097-1-Ig, 1:5,000)
- p21 (Huabio, Catalog No. HA500005, 1:1,000)
- Cyclin D1 (Huabio, Catalog No. ET1601-31, 1:1,000)
- Cyclin E1 (Huabio, Catalog No. ET1612-16, 1:1,000)
- Bcl-2 (GeneTex, Catalog No. GTX100064, 1:1,000)
- Bax (Huabio, Catalog No. ER0907, 1:1,000)
- Cleaved Caspase-3 (Proteintech, Catalog No. 25128-1-AP, 1:1,000)
- PI3K (Abmart, Catalog No. T40115, 1:1,000)
- p-PI3K (Abmart, Catalog No. T40065, 1:1,000)
- AKT (Proteintech, Catalog No. 60203-2-Ig, 1:5,000)
- p-AKT (Proteintech, Catalog No. 80455-1-RR, 1:5,000)
- SPP1 (Proteintech, Catalog No. 22952-1-AP, 1:1,000)

After primary antibody incubation, membranes were washed three times for 10 minutes each with TBST.

Subsequently, membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Proteintech, 1:5,000 dilution) for 1 hour at room temperature. Following three additional washes with TBST, protein bands were visualized using an enhanced chemiluminescence (ECL) detection kit (Beyotime, China, Catalog No. P0018FS) with a ChemiDoc XRS+ gel imaging system (Bio-Rad, USA). Densitometric analysis of protein bands was performed using ImageJ software (National Institutes of Health, USA), with band intensities normalized to β-actin.

Immunofluorescence Analysis

For immunofluorescence staining, KGN cells were seeded on sterile glass coverslips in 24-well plates. After specific treatments, cells were fixed with 4% PFA for 20 minutes at room temperature, followed by three washes with PBS. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at room temperature. Non-specific binding was blocked by incubating cells with 5% bovine serum albumin (BSA) in PBS for 30 minutes at room temperature.

Cells were then incubated with mouse anti-PCNA primary antibody (Proteintech, Catalog No. 60097-1-Ig, 1:500 dilution) overnight at 4°C. After three washes with PBS, cells were incubated with Alexa Fluor 594-conjugated goat anti-mouse secondary antibody (Proteintech, Catalog No. RGAM004, 1:200 dilution) for 2 hours at room temperature in the dark. Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole) for 5 minutes. Coverslips were mounted onto glass slides using an anti-fade mounting medium. Images were acquired using a fluorescence microscope (Thunder Imager, Leica, Germany).

Luciferase Reporter Assay

To investigate the transcriptional regulation of SPP1 by SOX3, luciferase reporter constructs were generated. The promoter region of human SPP1, including putative SOX3 binding sites, was amplified from human genomic DNA and cloned into the pGL3-Basic luciferase reporter vector (Promega, USA). Four deletion fragments of the SPP1 promoter were constructed: pGL3-s-1 (-1176 to +87 bp), pGL3-s-2 (-390 to +87 bp), pGL3-s-3 (-153 to +87 bp), and pGL3-s-4 (-25 to +87 bp). Based on JASPAR database predictions (http://jaspar.genereg.net/), three potential SOX3 binding sites (a, b, and c) were identified within the SPP1 promoter. Site-directed mutagenesis was performed on sites b and c within the pGL3-s-2 construct to generate mutant reporter plasmids (pGL3-s-2-b mut and pGL3-s-2-c mut). All constructs were verified by sequencing (Tsingke Biotech, Beijing, China).

KGN cells were seeded into 48-well plates and co-transfected with 400 ng of the respective SPP1 promoter luciferase reporter plasmid (wild-type or mutant) or the empty pGL3-Basic vector, along with 10 ng of pRL-TK Renilla luciferase plasmid (Promega) as an internal control for normalization of transfection efficiency. Cells were co-transfected with either 400 ng of pcDNA3.1-SOX3 overexpression plasmid or an empty pcDNA3.1 vector (pcDNA3.1-NC) using Lipofectamine 2000 (Invitrogen, Catalog No. 11668-019). After 6 hours, the serum-free medium was replaced with complete DMEM/F12. After 48 hours of incubation, luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, USA, Catalog No. E1910) and a Modulus Single Tube Multimode Reader (Turner Biosystems, USA). Relative luciferase activity was calculated by normalizing firefly luciferase activity to Renilla luciferase activity. Each transfection was performed in triplicate independent biological experiments.

Chromatin Immunoprecipitation (ChIP) Assay

Chromatin immunoprecipitation (ChIP) assays were performed to confirm the direct binding of SOX3 to the SPP1 promoter, as previously described [34]. Briefly, KGN cells were crosslinked with 1% formaldehyde (Sigma-Aldrich, Catalog No. F8775) in PBS for 10 minutes at room temperature to crosslink proteins to DNA. The crosslinking reaction was quenched by adding glycine to a final concentration of 0.125 M. Cells were then collected, lysed, and chromatin was sheared into fragments of 200-1000 bp by sonication using a Bioruptor Pico (Diagenode, USA).

Sheared chromatin lysates were pre-cleared with Protein G-agarose beads (Santa Cruz Biotechnology, Catalog No. Sc-2002) for 1 hour at 4°C. Pre-cleared chromatin was then incubated overnight at 4°C with either an anti-SOX3 antibody (Zen-bio, Catalog No. 862515) or a non-specific rabbit IgG antibody (Cell Signaling Technology, Catalog No. 2729) as a negative control. Immunoprecipitated complexes were captured by Protein G-agarose beads for 2 hours at 4°C. Beads were then washed sequentially with low-salt wash buffer, high-salt wash buffer, LiCl wash buffer, and TE buffer.

Chromatin was eluted from the beads, and crosslinking was reversed by incubation at 65°C overnight. DNA was then purified using the ChIP DNA Clean & Concentrator kit (Zymo Research, Catalog No. D5205). The enrichment of specific SPP1 promoter regions containing predicted SOX3 binding sites (site b and site c) in the immunoprecipitated DNA was quantified by RT-qPCR using specific primers (Supplementary Table S1). Results were expressed as fold enrichment relative to the IgG control.

Enzyme-linked Immunosorbent Assay (ELISA) Assay

The concentration of secreted SPP1 protein in cell culture supernatants was measured using a human SPP1 ELISA kit (Reed Biotech, Catalog No. RE1770H) according to the manufacturer's instructions. KGN cells were cultured in 12-well plates at a density of 1 × 10^5 cells/mL. After 36 hours of treatment, cell supernatants were collected, centrifuged to remove cell debris, and stored at -80°C until analysis. Standard curves were generated using recombinant human SPP1 provided in the kit. Absorbance was measured at 450 nm using a microplate reader. The

protein concentrations of the corresponding cell lysates were measured using the BCA protein assay kit (Beyotime, Catalog No. P0010) to normalize the secreted SPP1 levels to total cellular protein.

Bioinformatics Analysis

Transcriptome Sequencing and Differential Expression Analysis

Total RNA was isolated from OE-NC (n=3 biological replicates) and OE-SOX3 (n=3 biological replicates) KGN cells using TRIzol reagent (Invitrogen, Catalog No. 15596026) as described above. The quality and integrity of RNA samples were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). cDNA libraries were constructed following previously described methods [36]. Briefly, mRNA was purified from total RNA using oligo(dT) beads, fragmented, and then used as a template for first-strand cDNA synthesis. Second-strand cDNA was synthesized, followed by end repair, A-tailing, and ligation of sequencing adapters. The resulting cDNA libraries were then sequenced on an Illumina HiSeq 2500 platform (OE Biotech, Shanghai, China) using a 125 bp paired-end sequencing strategy.

Raw sequencing reads were pre-processed to filter out low-quality sequences, including reads containing adapters, reads with an unknown base ratio ≥ 10%, and reads with a low-quality base ratio (base quality ≤ 20) ≥ 50% using Trimmomatic software (version 0.39) [37]. The remaining high-quality clean reads were stored in FASTQ format. Clean reads were then mapped to the human reference genome (hg38) using HISAT2 software (version 2.2.1) [38], a fast spliced aligner designed for RNA-seq data. The alignment results were used to quantify gene expression levels. Fragment Per Kilobase of transcript per Million mapped reads (FPKM) values were calculated for each gene using the RSEM tool (version 1.3.1) [40], which provides accurate transcript quantification from RNA-Seq data.

Differentially expressed genes (DEGs) between OE-NC and OE-SOX3 KGN cells were identified using DESeq2 (version 1.30.1) in R, applying a statistical threshold of $|\log2(\text{Fold Change})| > 1$ (equivalent to a fold change (FC) > 2) and an adjusted p-value (q-value) < 0.05 to control for false discovery rate. Volcano plots and hierarchical clustering were generated to visualize the distribution and expression patterns of DEGs.

Gene Ontology (GO) and KEGG Pathway Enrichment Analysis

For functional annotation of DEGs, Gene Ontology (GO) enrichment analysis was performed using the WEGO 2.0 online tool (http://wego.genomics.org.cn/) [41]. GO terms are categorized into three main ontologies: molecular function (MF), cellular component (CC), and biological process (BP). GO terms with a p-value < 0.05 were considered significantly enriched.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was conducted to identify the biological pathways significantly associated with the DEGs. This analysis was performed using the KEGG public database (http://www.kegg.jp/) [42]. Pathways with a p-value < 0.05 were considered significantly enriched. Gene Set Enrichment Analysis (GSEA) was also performed using WebGestalt (WEB-based Gene SeT Analysis Toolkit, http://www.webgestalt.org/) to determine whether a predefined set of genes (e.g., genes in the PI3K/AKT pathway) was significantly enriched at the top or bottom of a ranked list of all genes, indicating a coordinated change in expression.

SOX3 Binding Site Prediction

Putative SOX3 binding sites within the SPP1 promoter region were predicted using the JASPAR database (http://jaspar.genereg.net/), a widely used open-access database of transcription factor binding profiles. The consensus motif for SOX3 binding was used to scan the SPP1 promoter sequence.

Statistical Analysis

All quantitative data are presented as the mean ± standard deviation (SD) from at least three independent biological replicates. Statistical analyses were performed using GraphPad Prism 8.0 software (GraphPad Software, USA). For comparisons between two groups, Student's t-test was employed. For comparisons involving more than two groups, one-way analysis of variance (ANOVA) was used, followed by Tukey's post-hoc test for multiple comparisons. A P-value < 0.05 was considered statistically significant. Specific statistical parameters (e.g., F-statistics, degrees of freedom) were recorded for each analysis.

RESULTS

SOX3 is Expressed in Granulosa Cells and Affects their Proliferation and Apoptosis

Our initial investigations aimed to confirm the presence and assess the functional impact of SOX3 in ovarian granulosa cells. Through quantitative real-time PCR (RT-qPCR) and Western blot analyses, we detected robust expression of SOX3 at both mRNA and protein levels in primary mouse granulosa cells and, more extensively, in the human KGN granulosa cell line. Further validation was obtained by re-analyzing publicly available RNA sequencing (RNA-seq) datasets derived from chicken and human granulosa cells during various stages of folliculogenesis [1, 2], which consistently demonstrated the presence of SOX3 transcripts, suggesting its conserved role in ovarian development across species.

To elucidate the functional role of SOX3, we manipulated its expression in KGN cells. We generated stable KGN cell lines overexpressing SOX3 (OE-SOX3) and control cells (OE-NC) using lentiviral transduction. As shown in Figure 1A and 1B, Western blot analysis confirmed a significant increase in SOX3 protein levels in OE-SOX3 cells compared to OE-NC. Similarly, RT-qPCR demonstrated a marked upregulation of SOX3 mRNA in OE-SOX3 cells (Figure 1C).

To assess the impact on cell proliferation, we performed the Cell Counting Kit-8 (CCK-8) assay. OE-SOX3 cells exhibited a significantly higher optical density (OD450 value) over a 72-hour period compared to OE-NC cells, indicating enhanced cell viability and proliferation (Figure 1D). This pro-proliferative effect was further corroborated by Western blot analysis of key cell cycle regulatory proteins. We observed a significant increase in the protein levels of Proliferating Cell Nuclear Antigen (PCNA), Cyclin D1, and Cyclin E1, while the cell cycle inhibitor p21 was notably reduced in OE-SOX3 cells compared to OE-NC cells (Figure 1E-I). Immunofluorescence staining for PCNA also revealed a higher number of PCNA-positive (proliferating) cells in the OE-SOX3 group (Figure 1J). Furthermore, the EdU incorporation assay, which directly measures DNA synthesis, showed a significant increase in the percentage of EdU-positive cells in SOX3-overexpressing KGN cells (Figure 1K and 1L), providing strong evidence for enhanced proliferative activity.

Conversely, to investigate the effects of SOX3 knockdown, we generated stable KGN cell lines with reduced SOX3 expression (sh-SOX3) using lentiviral shRNAs, with sh-NC as a control. Western blot and RT-qPCR analyses confirmed a significant reduction in both SOX3 protein and mRNA levels in sh-SOX3 cells compared to sh-NC cells (Figure 1M-O). Consistent with the overexpression results, SOX3 knockdown significantly inhibited KGN cell proliferation, as evidenced by lower CCK-8 absorbance values over 72 hours (Figure 1P). Western blot analysis revealed a significant decrease in PCNA, Cyclin D1, and Cyclin E1 protein levels, accompanied by a notable increase in p21, in sh-SOX3 cells compared to sh-NC cells (Figure 1Q-U). Immunofluorescence staining for PCNA and EdU incorporation assays further confirmed the inhibitory effect of SOX3 knockdown on cell proliferation (Figure 1V-X).

Beyond proliferation, we assessed the impact of SOX3 on granulosa cell apoptosis. Western blot analysis of apoptotic markers showed that SOX3 overexpression significantly increased the anti-apoptotic protein Bcl-2, while notably reducing the pro-apoptotic protein Bax and the active form of Caspase-3 (Cleaved Caspase-3) in OE-SOX3 cells compared to OE-NC cells (Figure 2A-D). The Terminal Deoxynucleotidyl Transferase-mediated dUTP-biotin Nick End Labeling (TUNEL) assay, which detects DNA fragmentation characteristic of apoptosis, revealed a markedly lower percentage of TUNEL-positive cells in SOX3-overexpressing cells (Figure 2E and 2F). Furthermore, flow cytometry analysis using Annexin V-APC/PI double staining confirmed a significant decrease in the overall apoptotic cell population (Annexin V-positive cells) in OE-SOX3 cells (Figure 2G and 2H). These results collectively indicate that SOX3 acts as a potent anti-apoptotic factor in KGN cells.

In contrast, knockdown of SOX3 in sh-SOX3 cells led to an increase in pro-apoptotic markers and a decrease in anti-apoptotic markers. Western blot analysis showed a significant increase in Bax and Cleaved Caspase-3, and a reduction in Bcl-2, in sh-SOX3 cells compared to sh-NC cells (Figure 2I-L). Both TUNEL assay and flow cytometry analysis consistently demonstrated a significant increase in the apoptotic rate in SOX3-knockdown KGN cells (Figure 2M-P). These findings strongly suggest that SOX3 plays a crucial role in suppressing apoptosis in granulosa cells, thereby contributing to their survival. The observed pro-proliferative and anti-apoptotic roles of SOX3 in granulosa cells are consistent with previous reports on SOX3's influence on ovarian granulosa cell proliferation [35] and its oncogenic properties in other cell types, such as osteosarcoma, where its downregulation inhibits proliferation and induces apoptosis [50].

Transcriptome Sequencing Data Analysis Reveals PI3K/AKT Pathway as a Key Target

To unravel the global transcriptional changes induced by SOX3 overexpression and identify potential regulatory mechanisms underlying its effects on proliferation and apoptosis, we performed comparative transcriptome sequencing (RNA-seq) of OE-NC and OE-SOX3 KGN cells. The sequencing yielded an average of 49.25 million and 49.29 million raw reads from OE-NC and OE-SOX3 cells, respectively. After stringent quality filtering using Trimmomatic [37], a high percentage of clean reads (approximately 98.9%) were obtained from both groups (Supplementary Table S2). These high-quality reads were then mapped to the human reference genome (hg38) using HISAT2 [38], with an average mapping rate exceeding 97% (Supplementary Table S3). Gene expression levels were quantified as FPKM values using RSEM [40].

Differential expression analysis identified a total of 2,957 differentially expressed genes (DEGs) between OE-SOX3 and OE-NC KGN cells, with a significance threshold of $|\log2(\text{Fold Change})| > 1$ and q-value < 0.05. Among these, 1,717 genes were significantly upregulated, and 1,240 genes were significantly downregulated in OE-SOX3 cells. A volcano plot visually represented the distribution of these DEGs, highlighting the genes with significant changes in expression (Figure 3A). Hierarchical clustering analysis further demonstrated distinct gene expression profiles between the OE-SOX3 and OE-NC groups, indicating a substantial transcriptional reprogramming induced by SOX3 overexpression (Figure 3B). The top 15 most significantly up-regulated and down-regulated DEGs were also identified and presented (Figure 3C).

To gain insights into the biological functions and pathways affected by SOX3, we performed Gene Ontology (GO) enrichment analysis on the identified DEGs using WEGO 2.0 [41]. The GO classification revealed that the DEGs were significantly enriched in various biological processes (BPs), including cellular process, metabolic process, and regulation of biological process. In terms of cellular components (CCs), enriched terms included membrane, membrane-enclosed lumen, and organelle. For molecular functions (MFs), categories such as binding, catalytic

activity, and transporter activity were significantly represented (Figure 3D). These broad categories highlight SOX3's pervasive influence on fundamental cellular activities.

Subsequently, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed using the KEGG public database [42] to identify the specific signaling pathways that were significantly altered in response to SOX3 overexpression. The analysis of upregulated DEGs revealed significant enrichment in several pathways, with the PI3K/AKT signaling pathway emerging as one of the most notably upregulated pathways in OE-SOX3 cells compared to OE-NC cells (Figure 3E). Other enriched pathways included Focal adhesion, ECM-receptor interaction, FoxO signaling pathway, Ras signaling pathway, and Hippo signaling pathway. Conversely, analysis of downregulated DEGs identified enrichment in pathways such as Cell cycle, p53 signaling pathway, DNA replication, and various metabolic pathways (Figure 3F). The strong enrichment of the PI3K/AKT signaling pathway in the upregulated gene set strongly suggested its critical involvement in mediating the pro-proliferative and anti-apoptotic effects of SOX3.

SOX3 Modulates the Activation of PI3K/AKT Signaling Pathway

Building upon the transcriptome analysis that implicated the PI3K/AKT pathway, we conducted further experiments to validate and characterize the regulatory relationship between SOX3 and this crucial signaling cascade. A comprehensive KEGG analysis of all DEGs confirmed the prominent enrichment of the PI3K/AKT signaling pathway among the altered genes (Figure 4A). Furthermore, Gene Set Enrichment Analysis (GSEA) provided compelling evidence for a significant positive enrichment of the PI3K/AKT signaling pathway gene set in SOX3-overexpressing KGN cells compared to control cells (Figure 4B). This indicates that the entire set of genes associated with the PI3K/AKT pathway was coordinately upregulated by SOX3.

To directly assess the activation status of the PI3K/AKT pathway, we measured the phosphorylation levels of key proteins in this cascade using Western blot analysis. In OE-SOX3 KGN cells, we observed a significant increase in the phosphorylation levels of both PI3K (p-PI3K) and AKT (p-AKT), while total PI3K and AKT protein levels remained largely unchanged (Figure 4C-E). This increased phosphorylation signifies enhanced activation of the PI3K/AKT pathway. Conversely, in KGN cells where SOX3 expression was stably knocked down (sh-SOX3), Western blot analysis revealed a notable reduction in the phosphorylation levels of both p-PI3K and p-AKT compared to sh-NC cells (Figure 4F-H).

To further validate these findings in an in vivo context, we examined the phosphorylation status of PI3K and AKT in ovarian tissues from sox3 knockout zebrafish compared to wild-type zebrafish. Consistent with our in vitro observations, the levels of p-PI3K and p-AKT were significantly decreased in the ovaries of sox3 knockout zebrafish (Figure 4I-K). These results strongly suggest that SOX3 positively regulates the activation of the PI3K/AKT signaling pathway in both human granulosa cells and zebrafish ovaries, aligning with the pathway's well-established roles in promoting cell survival, growth, and proliferation.

SOX3 Enhances Proliferation and Inhibits Apoptosis of KGN Cells Through PI3K/AKT Signaling Pathway

To definitively establish that the pro-proliferative and anti-apoptotic effects of SOX3 are indeed mediated through the PI3K/AKT signaling pathway, we employed a pharmacological inhibition strategy. We treated SOX3-overexpressing KGN cells (OE-SOX3) with LY294002, a well-characterized specific inhibitor of PI3K.

Western blot analysis confirmed that treatment with LY294002 significantly downregulated the protein expression levels of both p-PI3K and p-AKT in OE-SOX3 cells, effectively blocking the SOX3-induced activation of the PI3K/AKT pathway (Figure 5A-C). This indicated that LY294002 successfully abrogated the pathway's activity even in the presence of SOX3 overexpression.

Subsequently, we assessed the impact of PI3K/AKT pathway inhibition on cell proliferation and apoptosis in OE-SOX3 cells. The EdU incorporation assay revealed that the significant increase in proliferation rate observed in OE-SOX3 cells was markedly reversed when these cells were treated with LY294002 (Figure 5D-E). The percentage of EdU-positive cells in OE-SOX3 + LY294002 group was comparable to that of control cells, demonstrating that inhibiting PI3K/AKT signaling counteracted the proliferative effects of SOX3.

Similarly, the TUNEL assay showed that the suppressed apoptotic rate in OE-SOX3 cells was significantly increased upon treatment with LY294002 (Figure 5F-G). The percentage of TUNEL-positive cells in OE-SOX3 + LY294002 group was restored to levels similar to or even higher than control cells, indicating that blocking the PI3K/AKT pathway abolished the anti-apoptotic effects of SOX3. These compelling data unequivocally demonstrate that SOX3 promotes granulosa cell proliferation and inhibits apoptosis primarily through the activation of the PI3K/AKT signaling pathway.

SOX3 Regulates SPP1 Through Transcriptional Activation in KGN Cells

To identify the specific upstream factors through which SOX3 activates the PI3K/AKT signaling pathway, we revisited our transcriptome sequencing data, focusing on the significantly upregulated DEGs within the PI3K/AKT pathway. Secreted Phosphoprotein 1 (SPP1), also known as Osteopontin, emerged as the most significantly increased gene in OE-SOX3 KGN cells compared to OE-NC cells (Figure 6A). This finding was particularly intriguing

given SPP1's established role in activating PI3K/AKT signaling in various cellular contexts [60, 61, 62, 65] and its presence in ovarian granulosa cells [64].

To validate the RNA-seq results, we performed RT-qPCR and Western blot assays. Both analyses confirmed a significant upregulation of SPP1 at both mRNA and protein levels in KGN cells overexpressing SOX3 (Figure 6B-D). Conversely, stable knockdown of SOX3 in sh-SOX3 KGN cells resulted in a notable reduction in SPP1 mRNA and protein expression compared to sh-NC cells (Figure 6E-G). Furthermore, to confirm this regulatory relationship in vivo, Western blot analysis of ovarian tissues from sox3 knockout zebrafish showed a significant decrease in Spp1 protein levels compared to wild-type ovaries (Figure 6H and 6I).

Given that SPP1 is a secreted protein, we also measured its concentration in the cell culture supernatants using an Enzyme-linked Immunosorbent Assay (ELISA). The results revealed a significant increase in secreted SPP1 protein in the supernatant of OE-SOX3 KGN cells, while SOX3 knockdown led to a decrease in secreted SPP1 (Supplementary Figure S3). This suggests that SOX3 not only regulates intracellular SPP1 levels but also its secretion, potentially functioning as an autocrine/paracrine factor.

To investigate whether SOX3 directly regulates SPP1 transcription, we performed luciferase reporter assays. Based on predictions from the JASPAR database, we identified three putative SOX3 binding sites (designated a, b, and c) within the 5' flanking region of the human SPP1 promoter. We constructed a series of SPP1 promoter luciferase reporter plasmids with different deletion fragments and site-directed mutations. The relative luciferase activities of four deletion constructs (pGL3-s-1, pGL3-s-2, pGL3-s-3, pGL3-s-4) were measured. The results indicated that the region from -153 to -25 bp upstream of the SPP1 transcriptional start site was crucial for its transcriptional activity, as this region contained the predicted binding site 'c' and showed significant promoter activity (Figure 6N and 6O).

To further pinpoint the critical binding sites, we generated site-directed mutants for sites 'b' and 'c' within the pGL3-s-2 construct, as these sites were contiguous and shared similar SOX3 binding sequences (Figure 6P). Luciferase assays revealed that mutation of either site 'b' or site 'c' significantly decreased the promoter activity compared to the wild-type pGL3-s-2 construct. More importantly, overexpression of SOX3 significantly enhanced the luciferase activity driven by the wild-type pGL3-s-2 construct. While SOX3 overexpression could still upregulate the luciferase activity of the individual site 'b' or 'c' mutants, the effect was attenuated compared to the wild-type, suggesting that both sites contribute to SOX3-mediated activation (Figure 6Q).

To confirm the direct physical interaction between SOX3 and the SPP1 promoter, we performed a Chromatin Immunoprecipitation (ChIP)-qPCR assay. The results demonstrated that SOX3 significantly enriched the SPP1 promoter regions containing both site 'b' and site 'c', with a more pronounced enrichment at site 'c' (Figure 6R). These findings collectively provide strong evidence that SOX3 directly binds to and transcriptionally activates the SPP1 promoter in KGN cells, establishing SPP1 as a direct downstream target of SOX3.

SPP1 Mediates the Effects of SOX3 on Granulosa Cell Proliferation, Apoptosis, and PI3K/AKT Pathway

To unequivocally demonstrate that SPP1 is a critical mediator of SOX3's pro-proliferative and anti-apoptotic effects, and its regulation of the PI3K/AKT pathway, we conducted a series of rescue experiments. First, we confirmed the independent effects of SPP1 overexpression. Transient overexpression of SPP1 in KGN cells significantly promoted cell proliferation and inhibited apoptosis, accompanied by a notable increase in p-AKT levels (Figure 6A-C). This is consistent with the well-documented roles of SPP1 in promoting cell survival and growth in various contexts [59, 60, 61, 62, 64, 65].

Next, we performed rescue experiments by co-transfecting SOX3-overexpressing KGN cells (OE-SOX3) with siRNAs targeting SPP1 (si-SPP1-3). We found that knockdown of SPP1 in OE-SOX3 cells significantly attenuated the enhanced cell proliferation induced by SOX3 overexpression. The EdU incorporation rate in OE-SOX3 + si-SPP1 cells was significantly reduced compared to OE-SOX3 + si-NC cells (Figure 7A). Similarly, the anti-apoptotic effects of SOX3 overexpression were largely reversed by SPP1 knockdown, as evidenced by an increased apoptotic rate in OE-SOX3 + si-SPP1 cells compared to OE-SOX3 + si-NC cells (Figure 7B).

Crucially, we examined the impact on the PI3K/AKT pathway. Western blot analysis revealed that the SOX3-induced activation of the PI3K/AKT pathway, characterized by increased p-PI3K and p-AKT levels, was significantly reversed upon SPP1 knockdown in OE-SOX3 cells (Figure 7C). This indicates that SPP1 is essential for mediating SOX3's regulatory effects on the PI3K/AKT pathway.

Collectively, these rescue experiments provide robust evidence that SOX3 facilitates granulosa cell proliferation and suppresses apoptosis, at least in part, by directly upregulating SPP1 expression. SPP1, in turn, acts as a crucial upstream factor that activates the PI3K/AKT signaling pathway, thereby promoting the observed cellular phenotypes. This establishes a novel and critical SOX3-SPP1-PI3K/AKT regulatory axis in ovarian granulosa cells.

DISCUSSION

The intricate balance between granulosa cell proliferation and apoptosis is a cornerstone of successful folliculogenesis and, consequently, female reproductive health. This study has unveiled a novel regulatory

mechanism, demonstrating that the transcription factor SOX3 plays a pivotal role in promoting granulosa cell proliferation and inhibiting apoptosis. More significantly, we have elucidated the underlying molecular cascade, revealing that SOX3 exerts these effects by directly targeting and upregulating the expression of Secreted Phosphoprotein 1 (SPP1), which subsequently leads to the activation of the Phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling pathway. This newly identified SOX3-SPP1-PI3K/AKT axis represents a crucial regulatory node in ovarian granulosa cell dynamics.

Our findings that SOX3 enhances GC proliferation and suppresses apoptosis are consistent with emerging roles for SOX3 in various biological contexts, including ovarian function [35] and its established proliferative roles in other cell types [50]. While SOX3 has been extensively characterized for its fundamental roles in sex determination and neural development across diverse species [22, 25, 48, 49], its specific involvement in mammalian granulosa cell biology has remained less explored. Previous research in teleost fish, such as zebrafish, has highlighted the necessity of Sox3 for proper oogenesis and follicle development, where its deficiency leads to significant reproductive impairments, including follicular retardation and reduced fecundity [34]. Our study extends these crucial observations to mammalian granulosa cells, suggesting a conserved and vital pro-folliculogenic role for SOX3. The observed increase in PCNA, Cyclin D1, and Cyclin E1, coupled with a decrease in p21 upon SOX3 overexpression, provides a detailed molecular snapshot of how SOX3 promotes cell cycle progression. Conversely, the changes in Bcl-2, Bax, and Cleaved Caspase-3 levels clearly demonstrate its anti-apoptotic function, favoring cell survival.

The PI3K/AKT pathway is a universally recognized master regulator of cell survival, growth, and metabolism, with its activation being indispensable for promoting granulosa cell proliferation and preventing premature apoptosis, thereby supporting the overall integrity and development of ovarian follicles [3, 6, 7, 18, 20, 51, 52]. Our study provides robust evidence that SOX3 positively regulates the activation of the PI3K/AKT pathway in GCs, as demonstrated by increased phosphorylation of both PI3K and AKT upon SOX3 overexpression, and a reduction in their phosphorylation upon SOX3 knockdown, both in vitro and in vivo (in zebrafish ovaries). This direct link between SOX3 and the PI3K/AKT cascade is a critical finding, positioning SOX3 as an upstream regulator of a fundamental pro-survival signaling pathway in granulosa cells. The rescue experiments, where the SOX3-induced pro-proliferative and anti-apoptotic effects were abrogated by pharmacological inhibition of PI3K/AKT with LY294002, further solidify the notion that this pathway is indeed the primary mediator of SOX3's functions in granulosa cells. This aligns with studies showing that other factors influencing GC dynamics, such as the tyrosine phosphatase SHP2 and the deubiquitinating enzyme USP25, also modulate PI3K/AKT signaling to regulate follicular development [3, 7].

A cornerstone contribution of this research is the identification of Secreted Phosphoprotein 1 (SPP1) as a direct transcriptional target of SOX3 in granulosa cells. SPP1, also known as Osteopontin, is a multifunctional glycoprotein involved in a myriad of biological processes, including cell adhesion, migration, and survival. Its signaling capabilities are often mediated through interactions with cell surface receptors like integrins and CD44, leading to the activation of crucial downstream signaling pathways, including PI3K/AKT [59, 68]. The observation that SPP1 expression is upregulated in 3D cultures of rat ovarian granulosa cells via the PI3K/AKT pathway [64] further underscores its relevance in ovarian physiology. Our comprehensive data, including RT-qPCR, Western blot, and ELISA, consistently showed that SOX3 upregulates SPP1 expression at both mRNA and protein levels, and also increases its secretion. More importantly, the luciferase reporter assays and ChIP-qPCR provided compelling evidence of SOX3's direct binding to specific regions within the SPP1 promoter, leading to its transcriptional activation. This direct regulatory link between a transcription factor (SOX3) and a secreted signaling molecule (SPP1) is a novel finding that significantly enriches our understanding of the complex gene regulatory networks operating within granulosa cells.

The subsequent rescue experiments were pivotal in confirming the hierarchical relationship within this axis. We demonstrated that SPP1 overexpression alone could mimic the pro-proliferative and anti-apoptotic effects of SOX3, accompanied by PI3K/AKT activation. Crucially, when SPP1 expression was suppressed in SOX3-overexpressing cells, the beneficial effects of SOX3 on proliferation and apoptosis were significantly diminished, and the activation of the PI3K/AKT pathway was reversed. This unequivocally establishes SPP1 as a critical downstream effector that mediates SOX3's influence on granulosa cell dynamics and PI3K/AKT signaling. This finding is consistent with numerous studies demonstrating SPP1's role in activating PI3K/AKT in other pathological and physiological contexts, such as idiopathic pulmonary fibrosis, non-small cell lung cancer, and bladder cancer [60, 61, 62]. The mechanism likely involves SPP1 binding to its receptors on the granulosa cell surface, triggering intracellular signaling that converges on the PI3K/AKT pathway. As a secreted protein, SPP1 could also function in an autocrine or paracrine manner within the ovarian microenvironment, influencing neighboring granulosa cells or other ovarian cell types, thereby amplifying its pro-folliculogenic effects. This autocrine/paracrine loop of SPP1 has been observed in other contexts, such as breast cancer recurrence and lung cancer, where secreted OPN amplifies pro-tumorigenic effects [66, 67, 68].

While this study provides profound insights into the SOX3-

SPP1-PI3K/AKT axis in granulosa cell biology, certain limitations warrant consideration and suggest avenues for future research. Our experiments primarily utilized an immortalized human granulosa cell line (KGN) and primary mouse GCs. Although KGN cells are a valuable and widely accepted model for in vitro studies, they may not fully recapitulate the intricate complexity and heterogeneity of the in vivo ovarian microenvironment. Therefore, future studies should prioritize validating these findings in primary human granulosa cells from diverse patient populations and, more critically, in comprehensive in vivo mammalian models (beyond zebrafish, which, while informative for evolutionary conservation, differ significantly in reproductive biology from mammals). This would include conditional knockout or transgenic mouse models specifically targeting SOX3 in granulosa cells to directly assess its physiological role in folliculogenesis, ovulation, and fertility.

Furthermore, while we have identified SPP1 as a direct transcriptional target, the precise molecular mechanisms by which SOX3 binds to the SPP1 promoter (e.g., specific co-factors, chromatin modifications) could be explored in greater detail. Investigating potential feedback loops or crosstalk between the SOX3-SPP1-PI3K/AKT axis and other relevant ovarian signaling pathways, such as the Hippo pathway [15, 58], Ras signaling [54], FoxO signaling [55], or p53 signaling [56], which were also identified as enriched in our transcriptome analysis, would provide a more holistic understanding of granulosa cell regulation. The role of SPP1 as a secreted factor in mediating cell-cell communication within the follicle, potentially influencing oocyte-granulosa cell interactions or theca cell function, also merits further investigation. Finally, the clinical implications of dysregulated SOX3 or SPP1 expression in human ovarian disorders like PCOS or premature ovarian insufficiency warrant further translational research, potentially leading to novel diagnostic biomarkers or therapeutic strategies. For instance, modulating the activity of this axis could offer a new approach to enhance follicular development and improve female fecundity.

CONCLUSION

In conclusion, this study definitively identifies SOX3 as a novel and crucial regulator of granulosa cell proliferation and apoptosis. We establish a clear molecular cascade where SOX3 directly upregulates SPP1 expression, which in turn activates the PI3K/AKT signaling pathway, ultimately promoting granulosa cell survival and proliferation. This newly elucidated SOX3-SPP1-PI3K/AKT axis significantly enriches our understanding of the complex regulatory networks governing ovarian granulosa cell function and follicular development. Our findings provide a strong foundation for future investigations into the physiological and pathological roles of this axis and highlight its potential as a therapeutic target for improving female reproductive health.

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