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Androgen blockage impairs proliferation and function of Sertoli cells via *Wee1* and *Lfng*

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Abstract

Background Androgens are essential hormones for testicular development and the maintenance of male fertility. Environmental factors, stress, aging, and psychological conditions can disrupt androgen production, impacting the androgen signaling pathway and consequently spermatogenesis. Within the testes, testosterone is produced by Leydig cells and acts on Sertoli cells by activating the androgen receptor (AR), which then translocates to the nucleus to function as a transcription factor. Despite clinical correlations between low testosterone levels and diminished sperm quality, the precise mechanism remains unclear.

Methods This study explores the hypothesis that reduced androgen levels impair Sertoli cell function by disrupting AR transcriptional regulation. Using an androgen blockade model with enzalutamide, we investigated the impact of low androgen levels on AR target genes in Sertoli cells through ChIP-seq and RNA-seq assays.

Results Our results reveal that androgen blockage increases AR enrichment on the promoter region of *Wee1*, promoting *Wee1* expression, while decreasing binding to the promoter region of *Lfng*, inhibiting its expression. Increased WEE1 protein inhibits Sertoli cell proliferation, whereas reduced LFNG affects Notch modification, leading to decreased production of glial cell line-derived neurotrophic factor (GDNF), a key growth factor for spermatogonial stem cell self-renewal.

Conclusions These findings provide new insights into the molecular mechanisms by which low androgen levels interfere with Sertoli cell functions, offering novel perspectives for the clinical treatment of male reproductive disorders.

Keywords Cell cycle, Hypoandrogenism, Infertility, Microenvironment, Post-transcriptional modification

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Introduction

Androgens are pivotal hormones essential for testicular development and the maintenance of male fertility. Numerous studies have indicated that environmental pollutants, stress, aging, and psychological factors can alter androgen secretion within the testes, consequently affecting the androgen signaling pathway [1–3]. This disruption impacts the regulatory role of the testicular microenvironment on spermatogenesis, influencing both the quantity and quality of sperm. Clinically, a correlation between low testosterone levels and diminished sperm quantity has been reported [4, 5], though the precise relationship between androgen signaling and reduced sperm quantity remains largely unclear.

The androgen signaling pathway within the testicular microenvironment is complex. Testosterone is synthesized and secreted by Leydig cells, absorbed by Sertoli cells via diffusion, and binds to the androgen receptor (AR), causing a conformational change in the receptor protein, which then translocates into the nucleus to function as a transcription factor [6]. Preliminary studies have demonstrated AR expression in Sertoli cells from the neonatal period to adulthood in mice [7], highlighting the regulatory role of androgens throughout reproductive life. Although studies have underscored the essential role of androgen levels in testicular development and reproductive capability, the specific functions of androgens in Sertoli cells and the underlying molecular mechanisms remain to be elucidated.

Sertoli cells undergo two critical proliferation phases during testicular development: one in the neonatal period and another during puberty, around the first and third weeks, respectively [8]. This suggests a precisely regulated mechanism to ensure proper testicular development and function. Hormones such as GnRH and FSH are known to regulate Sertoli cell proliferation [8]. However, the specific role of androgen in Sertoli cell proliferation remains debated. Some studies suggest that AR signaling in Sertoli cells regulates the cessation of proliferation and the initiation of differentiation primarily through the Smad2/3 signaling pathway and down-regulation of AMH expression [9]. Conversely, other studies argue that androgens promote Sertoli cell proliferation, as AR knockout significantly reduces Sertoli cell numbers in a species- and stage-specific manner. Additional research indicates that androgens may promote Sertoli cell proliferation through the PI3K-AKT pathway by activating Ki67 and inhibiting CK18 [10]. Thus, the role of androgen signaling in regulating Sertoli cells remains elusive.

Cell cycle regulation is precisely fine-tuned through the balance of cell cycle drivers and inhibitors. Cyclin-dependent kinases (CDKs) promote cell proliferation, whereas their activity is regulated by WEE1 kinase, which directly phosphorylates and inhibits CDK1 and CDK2 [11]. WEE1 is an evolutionarily conserved genes from yeast to mammals [12, 13], encoding a tyrosine kinase regulates the G_2/M transition during cell cycle. An important target of WEE1 is cyclin-dependent kinase CDK1/Cyclin B-complex – the major driver of mitotic entry [14]. Therefore, increased WEE1 activity negatively regulates mitotic entry by deactivating the complex's ability to control critical cell cycle proteins, positioning WEE1 as a gatekeeper in regulating mitotic progress [15]. Notably, the proliferation capability of Sertoli cells is limited. In vivo, Sertoli cells exhibit only specific waves of proliferation throughout life, while primary Sertoli cells isolated from proliferating stages usually proliferate for only 2–3 passages in vitro, and those from non-proliferating stages manage even fewer passages [16, 17]. Thus, it is intriguing to unveil the regulatory mechanisms governing Sertoli cell proliferation.

Our preliminary work has shown that AR can bind to the promoter region of the *Gata2* gene in mouse Sertoli cells to regulate its expression. GATA2, in turn, binds to the enhancer of the key gene *Wt-1*, involved in Sertoli cell development, to regulate its expression [7]. This suggests that androgens promote *Wt-1* expression through *Gata2*, regulating testicular development. We have also identified ITGB1 [7] and Connexin 43 [18] as direct binding targets of WT1 in Sertoli cells, key membrane proteins involved in recognizing spermatogonial stem cells (SSCs) and regulating their binding and homing to the microenvironment and gap junction in blood-testis-barrier, respectively. Hence, low androgen levels can directly interfere with AR activity, affecting its binding targets in Sertoli cells.

Additionally, paracrine signaling is a crucial function of Sertoli cells. Glial cell line-derived neurotrophic factor (GDNF), secreted by Sertoli and peritubular myoid (PM) cells [19, 20], maintains SSC self-renewal. GDNF deficiency leads to the loss of SSCs, causing azoospermia. Existing studies have shown that GDNF production in Sertoli cells is regulated by the Notch signaling pathway. NOTCH receptors (NOTCH1-4) are activated by contact with membrane-bound ligands on neighboring cells, such as JAGGED (JAG) and DELTA (DLL). Activation of the canonical pathway leads to cleavage and nuclear translocation of the NOTCH intracellular domain (NICD), which associates with the DNA-binding protein RBPJ to activate transcriptional repressors HES1 and HEY1, directly repressing GDNF expression in Sertoli cells. High expression of the NOTCH ligand JAG1 by undifferentiated spermatogonia, compared to other germ cells, suggests these cells participate in the activation of

NOTCH signaling and GDNF modulation within the stem cell niche [21]. Our preliminary research supports this, showing that ZEA activates the Notch signaling pathway in Sertoli cells, thereby inhibiting GDNF production, underscoring the crucial role of GDNF in SSC maintenance. Additionally, testosterone regulates GDNF expression in PM cells to support SSC maintenance [20]. Both Sertoli and PM cells robustly express AR from neonatal to adult testis, implying that androgen signaling might regulate GDNF production in Sertoli cells. However, the specific regulatory mechanisms require further investigation.

The *Lfng* gene, belonging to the glycosyltransferase family, can elongate O-linked fucose residues on Notch, altering Notch signaling [22]. Knockdown of *LFNG* in human Kras-induced pancreatic ductal adenocarcinoma cell lines activates Notch signaling [23], suggesting that LFNG inhibits Notch activity. Notably, modification by LFNG decreases JAG1 binding to NOTCH2 but not DLL1[24], indicating a regulatory mechanism of LFNG on Notch signaling. Based on this evidence, we propose that androgen signaling regulates Sertoli cell proliferation and GDNF production, and the underlying molecular mechanism merits investigation.

Given that low androgen levels, both in animal models and clinically, impede testicular development and function, we hypothesize that reduced androgen levels may significantly disrupt Sertoli cell proliferation or function by disturbing the AR's transcriptional regulation. To investigate this hypothesis, a pharmacological blockade model of AR signaling is required. Clinically, enzalutamide is widely used in treating castration-resistant prostate cancer [25]. With its high affinity for AR, enzalutamide can block androgen signaling, potentially disturbing the target genes of AR as a transcription factor [26]. Therefore, we employed an androgen drug blockade model, and utilized ChIP-seq and RNA-seq to investigate the changes in downstream targets of AR in Sertoli cells and the corresponding expression profiles, to elucidate the impact of decreased androgen levels on Sertoli cell function. Our results indicate that enzalutamide's competitive binding to AR alters the expression of genes involved in cell cycle and Notch signaling. Molecular evidence further elucidates the regulatory mechanisms through which androgen signaling affects Sertoli cell proliferation and GDNF production. These findings provide novel insights into the molecular mechanisms through which low androgen levels affect male fertility, offering new perspectives for the clinical treatment of male reproductive disorders.

Materials and methods

Animals and ethics statement

Male ICR mice were obtained from the Comparative Medicine Centre of Yangzhou. All procedures involving animals were conducted in strict accordance with the ethical standards and approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Agricultural University.

Isolation of SCs

SCs were extracted from testes of seven days postpartum (dpp) mice following previous protocol [27]. Briefly, the testes were dissected, and the seminiferous tubules were collected and subjected to digestion with 1 mg/mL collagenase IV to remove Leydig cells. Subsequently, the tubules were further digested with 1 mg/mL collagenase IV to dissociate peritubular cells. The digestion process was carefully monitored under a microscope. The single-cell suspension obtained through trypsin digestion was subsequently blocked with fetal bovine serum (FBS). The resulting cell pellet was resuspended with SCs medium and cultured at 37 °C with 5% CO_2 for 12 h, and finally a hypotonic treatment was performed to eliminate germ cells.

SCs culture and treatment of enzalutamide or dihydrotestosterone (DHT)

Enzalutamide (Selleck, S1250) was solved in DMSO to a concentration of 5 or 10 μM. DHT was prepared in anhydrous ethanol to a concentration of 10 nM [7]. Purified Sertoli cells were seeded into a 24-well plate to a final density of 1.5×10^5 cells per well and subsequently treated with dihydrotestosterone (DHT) and/or enzalutamide, respectively. Following a 48-h incubation period, the SCs were harvested for subsequent analyses.

The culture medium for SCs consisted of DMEM/F12 media, 10% FBS, 2 mM L-Glutamine (Sigma, Cat. No. G7513), 100 μg/ml penicillin (Aladdin, Cat. No. P105489), and 1 μM sodium pyruvate (Sigma, Cat. No. P2256).

RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from testicular tissue or SCs using TRNzol Reagent (Tiangen, Cat. No. DP405) and reverse transcribed into cDNA with the PrimeScript™ RT Reagent Kit (TAKARA, Cat. No. RR036A). Primer sequences are detailed in Supplementary Table S1. GAPDH served as the normalization control for quantitative analyses.

Immunofluorescence (IF)

The IF assays were performed following previous protocol [28]. Briefly, SCs were fixed with Carnoy's solution (methanol:acetic acid, 3:1) for 20 min at -20 °C. Following a phosphate-buffered saline (PBS) wash, samples were blocked in 10% goat serum for 10 min at 37 °C and sequentially incubated with primary and secondary antibodies (refer to Supplementary Table S2 for antibody details). Nuclei were counterstained with DAPI. Fluorescence images were acquired post-staining.

Western Blot (WB) analysis

The WB assays were performed following previous protocol [28]. Briefly, Cell lysates were prepared using RIPA buffer (Shanghai Yeasen Biotechnology Co., Ltd.) supplemented with a protease inhibitor cocktail. Protein samples were resolved on SDS-PAGE gels of appropriate concentrations (6%, 8%, and 12%) and transferred onto nylon membranes. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated with primary antibodies overnight at 4 °C. Following TBST washes, membranes were incubated with secondary antibodies at room temperature for 1 h. Enhanced chemiluminescence was employed for detection.

ChIP-seq and ChIP-qPCR

The library preparation for Chromatin Immunoprecipitation Sequencing and data analysis were performed as previous protocol with minor modification [28].

To initiate the construction of a ChIP-seq library, Sertoli cells underwent a crosslinking step using 1% formaldehyde for a duration of 10 min at ambient temperature. This reaction was subsequently quenched with 125 mM glycine. The chromatin was fragmented, and the resulting fragments were subjected to a pre-clearing process. Immunoprecipitation was carried out using magnetic beads conjugated to both Protein A and Protein G, which were pre-bound to antibodies targeting the anti-Androgen Receptor antibody (Millipore, 06–680). Following the immunoprecipitation, the crosslinks were reversed, and the DNA, both from ChIP and input samples, was processed through end-repair and A-tailing using the NEBNext End Repair/dA-Tailing Module (E7442, New England Biolabs). This was followed by the ligation of adaptors utilizing the NEBNext Ultra Ligation Module (E7445, New England Biolabs). The resulting DNA libraries were then subjected to 15 cycles of amplification and sequenced on the Illumina Hi-Seq platform, employing a paired-end 2 × 150 bp sequencing approach.

The initial step in data analysis involved the refinement of raw sequencing reads to secure high-quality, clean reads. This was achieved by the elimination of sequencing adapters, the exclusion of short reads (length < 35 bp), and the removal of low-quality reads. The software Cutadapt v1.18. was employed with specific parameters (–max-n 0 –minimum-length 35) to perform these tasks, followed by the use of Trimmomatic v0.38 with adjusted parameters (SLIDINGWINDOW:4:15 LEADING:10 TRAILING:10 MINLEN:35). Subsequently, FastQC was utilized under default settings to verify the quality of the reads. The high-quality reads were aligned to the mouse reference genome (assembly GRCm38) using Bowtie2 v2.3.4.1 with standard parameters. Duplicate reads were identified and removed with the aid of Picard MarkDuplicates. The MACS v2.1.2 algorithm, with parameters set to identify peaks at a significant threshold (p-value cutoff of 0.01), was employed for peak detection. Finally, the ChIPseeker R package was utilized for the annotation of identified peak regions to corresponding gene features, thereby facilitating the biological interpretation of the ChIP-seq data. To download the original data, please visit the GEO database website at https://www.ncbi.nlm.nih.gov/gds/ and use the code: PRJNA1140838.

ChIP-qPCR

The qPCR amplification was performed using the 2 × Universal Blue Multiplex Probe qPCR Master Mix from Saville Company. The reaction volume was set to 25 μL, comprising the following components: 12.5 μL of 2 × Universal Blue Multiplex Probe qPCR Master Mix, 1 μL of 10 μmol/L Forward Primer, 1 μL of 10 μmol/L Reverse Primer, and approximately 20 ng of DNA template, with deionized water added to bring the total volume to 25 μL. The mixture was homogenized before being loaded. Upon completion of the reaction, the NC was used as the internal reference, and the fold change was calculated using the 2-ΔΔCt method. The primer sequences were as follows:

Wee1 (F:5'-GGTTGGACCAGGCTTCTAGG-3', R:5'-CCCCACCCAGAGTCTATGTT-3'),
Lfng (F:5'-GTGTCCGGATGCTTCTGAAC-3', R:5'-ATGTTTAGAGGACCGGAGCC-3'),
NC (F:5'-ATTGCCGGTTTCTTGCAGTG-3', R:5'-GGGGGCTACCTAAGCAACAA-3').

RNA-seq

RNA-seq assay was performed as previous protocol with minor modification [29]. For RNA-seq library preparation, total RNA was extracted using Trizol and purified from genomic DNA using DNase, with mRNA subsequently isolated via the NEBNext PolyA module and libraries constructed using the NEBNext Ultra

Directional kit for Illumina sequencing (2×150 paired-end). Quality control entailed adapter trimming and filtering for high-quality reads with Cutadapt and Trimmomatic, and quality assurance with FastQC, followed by alignment to the human GRCh38 genome using HISAT2. Differential expression was analyzed using StringTie and edgeR, considering genes with an adjusted P-value < 0.05 and $|\log2FC| \geq 1$ significant. Functional enrichment for GO terms and KEGG pathways was performed using ClusterProfiler with gene annotations from Ensembl database, streamlining the process from RNA preparation to functional analysis. To download the original data, please visit the GEO database website at https://www.ncbi.nlm.nih.gov/gds/ and use the code:PRJNA1140838.

Oligonucleotide transfection

Primary SCs were plated on 24-well plates for 48 h and grown to a 70%-80% confluence. Oligonucleotides were then transfected into the SCs at a final concentration of 1 μg using RNAimax (Invitrogen, L3000150) for 48 h, after which the SCs and their conditioned medium were collected for further analysis. The siRNA sequences are provided in Table S1.

Statistical analysis

Randomly selected sections or immunofluorescence fields were subjected to cell counting. Data were processed using Excel and are presented as mean ± standard deviation (SD). Statistical significance was assessed using Student's t-test and analysis of variance (ANOVA).

Results

Establishment of AR pharmacological blockage model in Sertoli cells through enzalutamide treatment

To obtain purified Sertoli cells, 7-day male mice were sacrificed to collect testes, and isolated Sertoli cells were purified after enzyme digestion and hypotonic treatment (Fig. 1A and B). Immunofluorescence of AR and WT1 confirmed Sertoli cell identity and purity (WT1 + ratio is 87.4 ± 1.2%, AR + ratio is 93.1 ± 1.4%) (Fig. 1C-E). Cells were identified by RT-PCR, showing that Sertoli cell marker *Ar, Sox9, Fshr* and *Wt-1* were detected at mRNA levels, whereas germ cell marker MVH marker was not detected (Fig. 1F). These results suggested that Sertoli cells were efficiently enriched. Purified Sertoli cells were treated with vehicle (DMSO) or enzalutamide as Method section described, and the relative expression levels of *Ar* and its downstream target gene *Rhox5* [30] were significantly declined, implying that AR signaling was inhibited by enzalutamide in a dosage of 10 μM (Fig. 1G). This result implied that an in vitro model mimicking AR signal inhibition in Sertoli cells was established.

Screening the differential target genes of AR in DMSO and enzalutamide treated Sertoli cells using ChIP-seq assay

To reveal the androgen receptor's differential downstream target genes and transcriptional profiles across two distinct cohorts, the strategy for Sertoli cells isolation, treatment, and analysis was exhibited (Fig. 2A). Sertoli cells treated with DMSO or 10 μM enzalutamide were harvested for ChIP-seq and RNA-seq assays, respectively. According to ChIP-seq results, a comprehensive set of 1,200 enriched regions was delineated, with genes located within these domains undergoing subsequent evaluation (Table S3). This included clustering according to Gene Ontology (GO) and exploration of potential pathways via Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis.

The spatial distribution of AR-binding peaks was depicted in Fig. 2B. Notably, treatment with enzalutamide was associated with an augmented propensity of AR to associate with promoter regions, with an increase from 17.8% in the vehicle-treated group to 22.2% in the enzalutamide-treated group, suggesting that the change of AR activity affects its binding priority on genomes and might directly disturb gene expression.

The results of ChIP-seq revealed that under physiological conditions and following enzalutamide treatment, AR-bound target genes are enriched in cell division, phosphorylation, regulation of circadian rthythm, chromatin remodeling, DNA repair, cell differentiation, etc., through Biological Process (BP) analysis (Fig. 2C), suggesting that AR signal regulate multiple functions in Sertoli cells. However, after androgen blockade, an enrichment of AR target genes was observed in the In utero embryonic development, regulation of transcription, DNA-templated, protein transport, chromatin remodeling, regulation of translation, regulation of cell cycle, etc. (Fig. 2D). We observed that under conditions of androgen blockade, there is a partial alteration in the categories of downstream target genes of AR. Notably, certain biological functions such as RNA splicing and cell cycle regulation remain under AR regulation, suggesting that these functions are of substantial importance and continue to be regulated even under conditions of low AR activity. Subsequently, we analyzed the BP pathways of differential target genes between the control and enzalutamide treated groups (Fig. 2E), and noticed that the differential genes are still involved in the regulation of apoptosis and the cell cycle. However, the name of gene cluster regarding cell proliferation is "negative regulation of cell population proliferation", implying that the specific genes bound and regulated by AR, as well as their biological functions, might differ after androgen blockage. Thus, we hypothesize that the AR may have different roles in cell cycle regulation depending on androgen

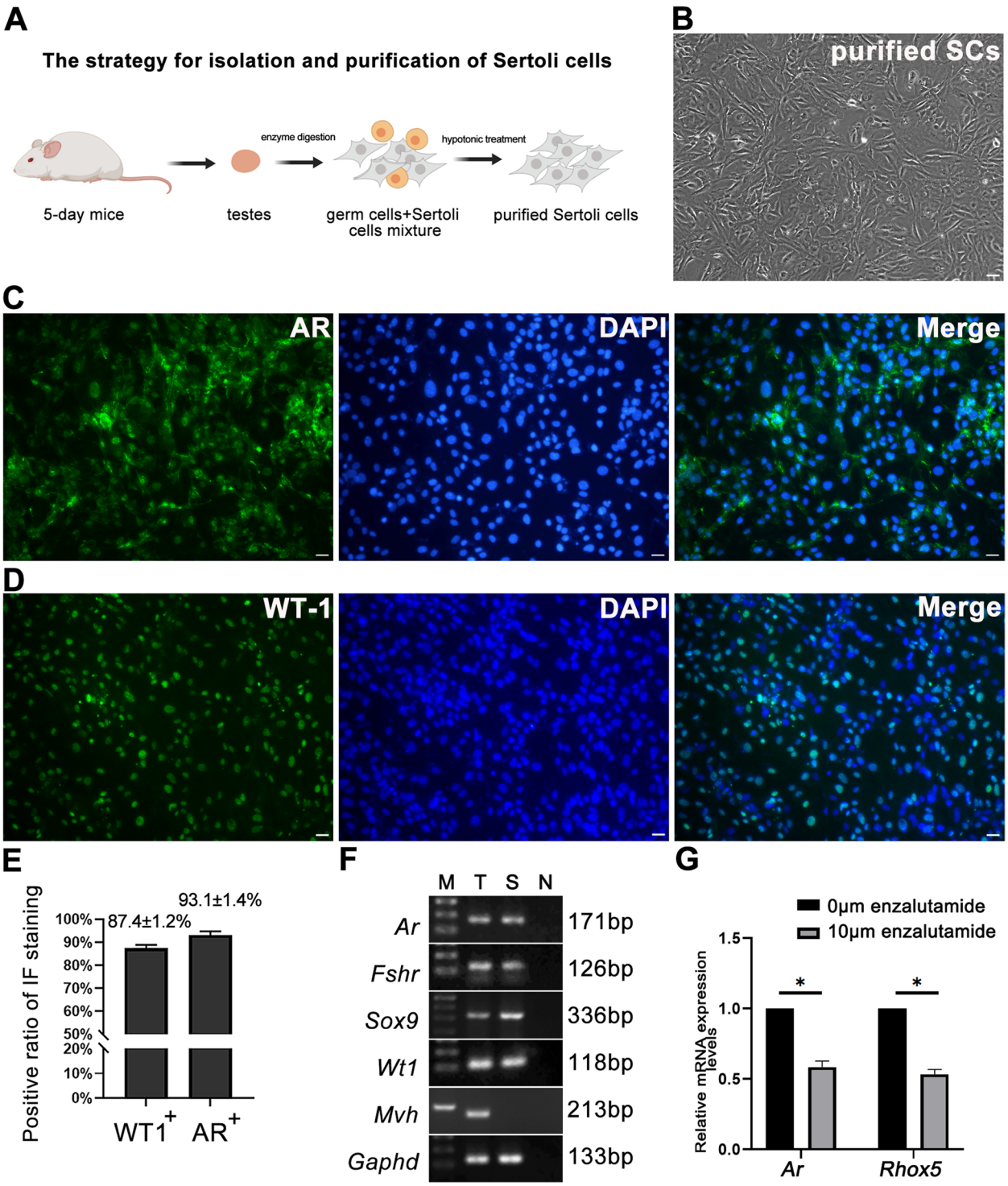

Fig. 1 Isolation of Sertoli cells and establishment of androgen deprivation model. The strategy of Sertoli cell isolation and purification (**A**). Morphology of purified Sertoli cells were exhibited (**B**). IF staining was used to detect AR (**C**) and WT1 (**D**) in purified Sertoli cells, and the positive ratios were statistically exhibited (**E**). The expression levels of *Ar*, *Fshr*, *Sox9*, *Wt1* and *Gapdh* in purified Sertoli cells were detected using RT-PCR (F). The relative expression levels of *Ar* and *Rhox5* compared to β-tubulin were determined in Sertoli cells treated with DMSO or 10 μM enzalutamide (**G**). Results are presented as mean ± SD, with significance levels indicated by n.s. $p > 0.05$, *$p < 0.05$, **$p < 0.01$. Scale bar = 20 μm

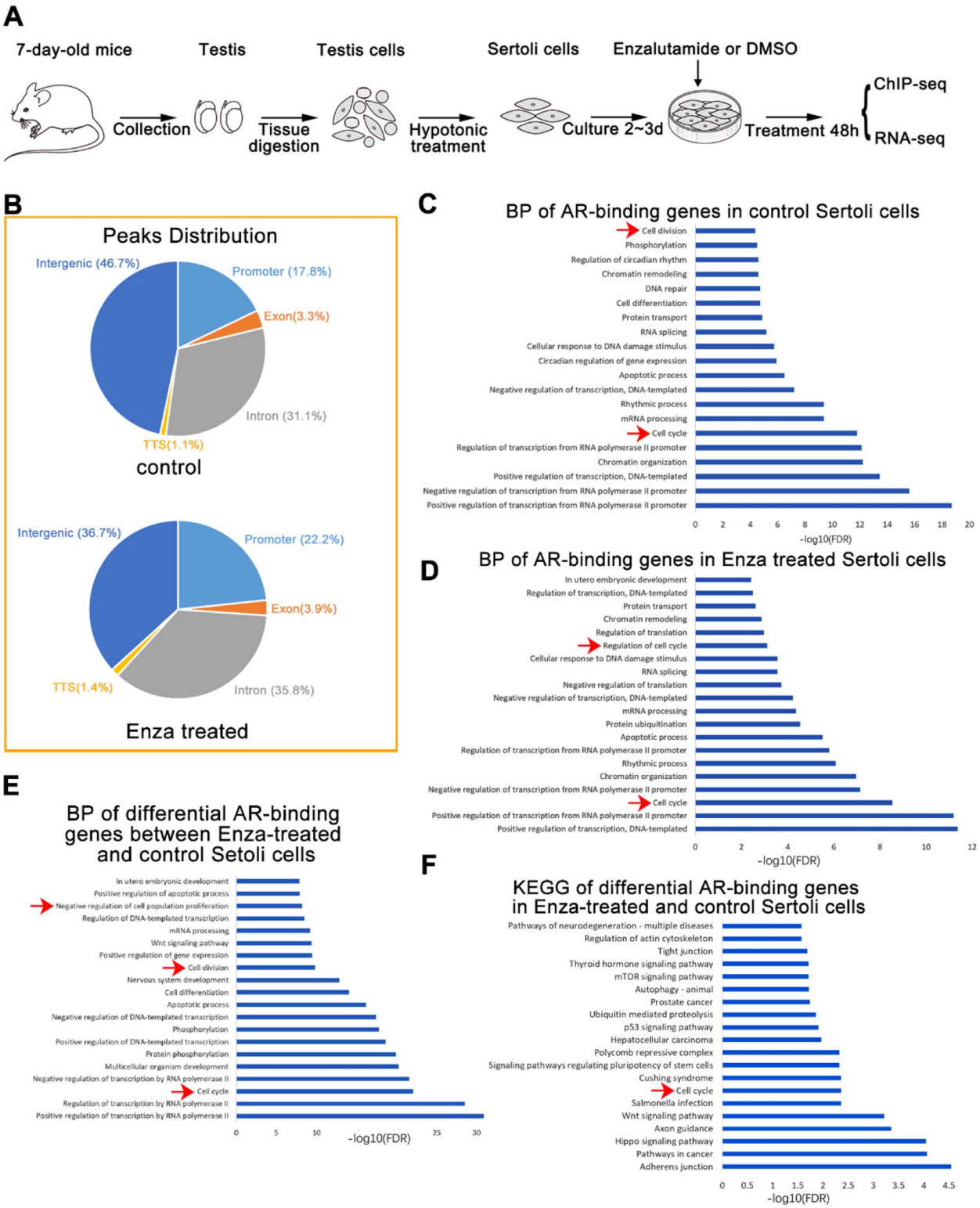

Fig. 2 Comparison of AR binding regions in vehicle- and enzalutamide-treated Sertoli cells. **A** Experimental strategies for androgen blockage and ChIP-seq assay are depicted. **B** Peak distribution of AR in vehicle- and enzalutamide-treated Sertoli cells is shown. **C**, **D** GO pathway analyses of genes with differential AR enrichment in vehicle- and enzalutamide-treated Sertoli cells, respectively. **E** Differential AR binding genes in vehicle- and enzalutamide-treated Sertoli cells are illustrated via GO pathway analysis. **F** KEGG pathway analysis of genes with differential AR enrichment in vehicle- and enzalutamide-treated Sertoli cells

levels. According to the KEGG pathway analysis, we found that in addition to the cell cycle, multiple signaling pathways, including mTOR, p53, and Wnt, were enriched (Fig. 2F). This indicates that these pathways might also be involved in the AR signaling pathway in Sertoli cells. However, the potential connection of altered biological processes and signaling pathways after androgen blockage need to be investigated.

Comparison of transcriptomes of enzalutamide or DMSO treated Sertoli cells

To further reveal the impact of androgen blockage on gene expression pattern, we analyzed the differential expressed genes (DEGs) using RNA sequencing assay, and identified 509 DEGs in Sertoli cells treated with enzalutamide compared to those under normal physiological conditions, with 219 genes upregulated and 290 downregulated (Fig. 3A) (Table S4). A heatmap illustrated the transcriptional divergence between wild-type and enzalutamide-treated Sertoli cells (Fig. 3B). Pathway enrichment analyses via KEGG and GO suggested that attenuation of AR signaling perturbed extracellular matrix interactions and biosynthetic processes (Fig. 3C and D). Notably, alterations in cytochrome P450 genes indicated that AR inhibition disrupted the cholesterol to pregnenolone conversion pathway [31]. Gene Set Enrichment Analysis (GSEA) further delineated the molecular consequences of AR blockade, highlighting the upregulation of pathways related to mitochondrial protein signaling, cholesterol, and sterol biosynthesis, while pathways

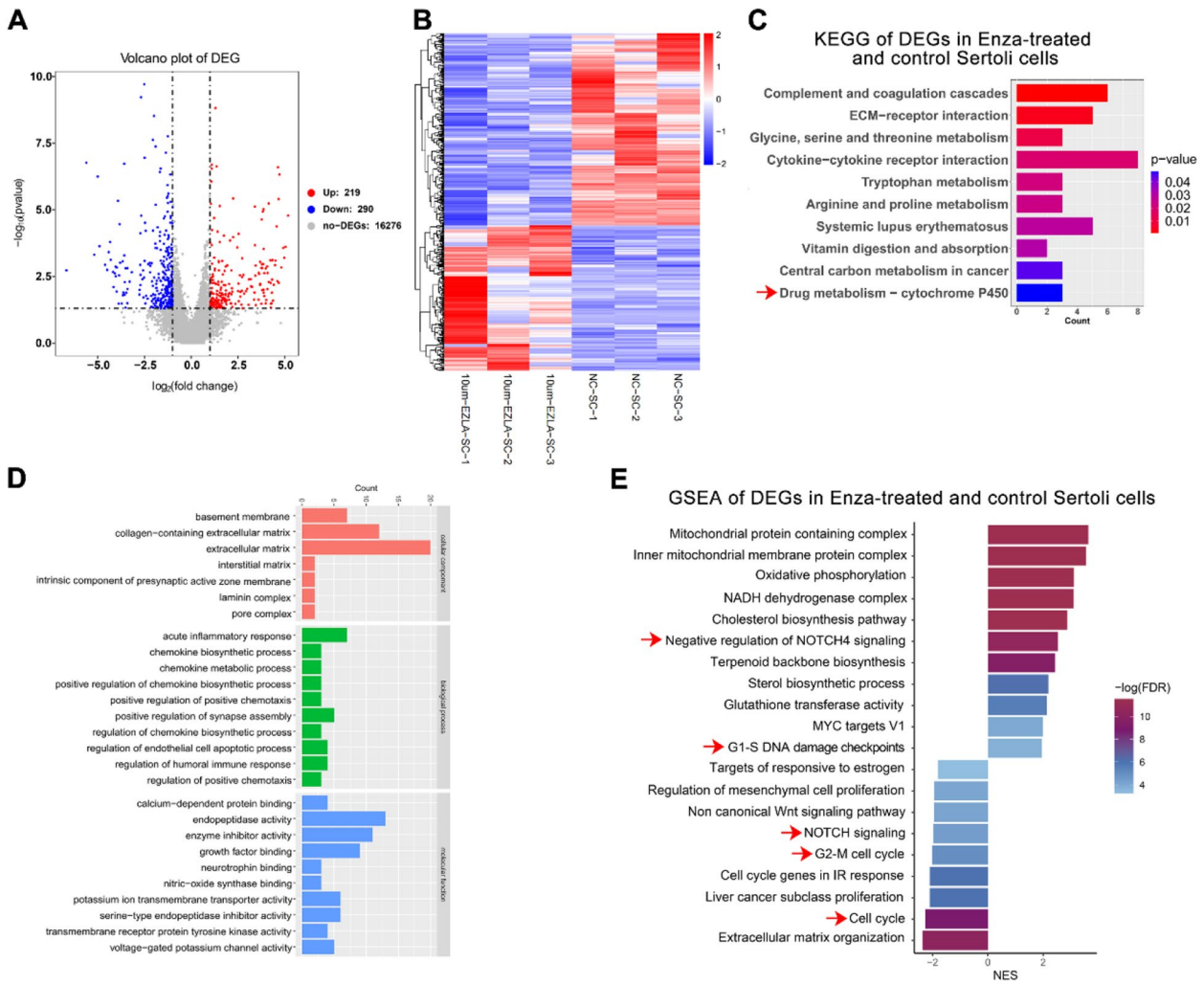

Fig. 3 Analysis of differentially expressed genes (DEGs) in vehicle- and enzalutamide-treated Sertoli cells. **A** Volcano plot and (**B**) heatmap displaying DEGs between vehicle- and enzalutamide-treated Sertoli cells. **C** KEGG pathway analysis and (**D**) GO analysis of DEGs in the respective groups. **E** GSEA analysis demonstrating expression changes in key signaling pathways in vehicle- and enzalutamide-treated Sertoli cells

linked to estrogen response, Notch, non-canonical Wnt signaling, and cell cycle progression were suppressed post-enzalutamide treatment (Fig. 3E). These findings suggest a compensatory upregulation of genes involved in androgen synthesis in Sertoli cells and a concomitant reduction in proliferative and functional capacity, potentially through downregulation of non-canonical Wnt and Notch signaling pathways—aligning with the KEGG results from ChIP-seq assays and underscoring androgen's critical role in Sertoli cell proliferation and function. Additionally, our recent work indicated that hyperactive Notch signaling curtailed GDNF production, leading us to hypothesize that AR inhibition may similarly impact GDNF synthesis in Sertoli cells.

Integrative analysis revealed that *Wee1* and *Lfng* are two key target genes of AR affected by androgen blockage

To further reveal the impact of androgen blockage on gene expression, the integrative analysis of ChIP-seq and RNA-seq datasets was performed. The results revealed increased AR binding at the *Wee1* locus, correlating with heightened *Wee1* expression, while AR occupancy and expression of *Lfng*, key regulators of Sertoli cell proliferation and function, were diminished (Table S5). These findings indicate that *Wee1* and *Lfng* as two direct binding targets of AR in Sertoli cells. To further analyze the binding of AR to *Wee1* and *Lfng* genes and regulatory effect on their transcription, we examined the enrichment of AR on the sequences of *Wee1* and *Lfng* genes through IGV analysis. It was observed that under androgen signal blockade, the enrichment of AR on *Wee1* promoter region remarkably increased, whereas its enrichment on *Lfng* noticeably decreased (Fig. 4A). To further validate this result, we performed ChIP-qPCR to detect the enrichment regions of AR at the *Wee1* and *Lfng* genes. The qPCR results showed a significant increase in AR enrichment at *Wee1* and a significant decrease at *Lfng* (Fig. 4B), consistent with the IGV findings. Moreover, we employed RT-PCR to assess the expression differences of *Wee1* and *Lfng* between the control group and the group treated with 10 μM enzalutamide (Fig. 4C). The results indicated that 10 μM enzalutamide not only significantly inhibited AR expression but also led to an increase in *Wee1* expression and a decrease in *Lfng* expression. Additionally, the expression level of *Gdnf* also decreased. Since *Gdnf* might be regulated by the LFNG signaling pathway, these findings further confirm that under androgen blockade, the regulation of the two key genes involved cell proliferation and secretion of GDNF, *Wee1* and *Lfng*, might be directly influenced by AR.

To validate the transcriptome results, we selectively examined some key genes regulating cell proliferation. The results showed a decrease in the expression of *Cdk1*, *Cdk2*, *Ccnd1*, *Ccnd3*, and *Pcna*, indicating a reduction in cell proliferation capacity following AR blockade (Fig. 4D). Subsequently, we investigated the expression changes in key genes of two crucial signaling pathways related to cell proliferation, namely, the Wnt and JAK-STAT pathways. We found that upon enzalutamide treatment of Sertoli cells, although *Fzd1*, *Fzd2*, *Dkk3*, and *Cacybp* were downregulated, the core genes of the Wnt signaling pathway such as *Apc*, *β-catenin*, and *Axin2* showed no significant changes (Fig. 4E), suggesting no critical relationship between AR signaling and the core Wnt pathway. However, a significant decrease was observed in the core genes of the JAK-STAT signaling pathway, including *Jak2*, *Stat1*, *Stat3*, *Pias1*, *Pias2*, and *Socs2* (Fig. 4F), implying a connection of cell proliferation mediated by androgen and JAK-STAT signaling pathway. To further verify this result, Sertoli cells treated with DMSO or 10 μM enzalutamide were harvested for Western blotting. Androgen blockage by enzalutamide led to up-regulated WEE1, and down-regulated AR, PCNA, JAK2 and STAT, as well as decreased phosphorylation of STAT3 (Fig. 4G and H). This indicates that androgen blockade can directly impact the JAK-STAT pathway, potentially serving as a key mechanism leading to the decline in Sertoli cell proliferation.

Blockage of AR signaling pathway inhibited proliferation of Sertoli cells via *Wee1*

To discern whether the upregulation of WEE1 expression induced by androgen blockade is a pivotal factor in inhibiting Sertoli cell proliferation, we embarked on

(See figure on next page.)

Fig. 4 Identification of AR's target genes *Wee1* and *Lfng* in Sertoli cells. **A** IGV snapshots showing AR binding peaks in *Wee1* and *Lfng* genes. **B** Enrichment of AR at the promoter regions of *Wee1* and *Lfng* confirmed by ChIP-qPCR. **C** Relative expression levels of *Wee1*, *Lfng*, *Gdnf*, and *Ar* in Sertoli cells treated with 0 and 10 μM enzalutamide, as determined by RT-PCR. **D** RT-PCR analysis of proliferation-associated genes (*p19*, *p21*, *Cdk1*, *Cdk2*, *Ccnd1*, *Ccnd3*, *Pcna*) in Sertoli cells treated with DMSO or 10 μM enzalutamide. **E** Relative expression levels of genes in the Wnt signaling pathway (*Apc*, *Apc2*, *β-catenin*, *Axin2*, *Fzd1*, *Fzd2*, *Dkk3*, *Cacybp*) in DMSO or 10 μM enzalutamide-treated Sertoli cells, determined by RT-PCR. **F** Relative expression levels of genes in the JAK-STAT signaling pathway (*Jak2*, *Stat1*, *Stat3*, *Pias1*, *Pias2*, *Socs2*) in DMSO or 10 μM enzalutamide-treated Sertoli cells assessed by RT-PCR. **G** Protein levels of AR, WEE1, PCNA, JAK2, STAT3, and phosphorylation of STAT3 in DMSO or 10 μM enzalutamide-treated Sertoli cells detected by Western blotting. **H** Statistical representation of Western blotting results. Results are presented as mean ± SD, with significance levels indicated by n.s. $p > 0.05$, *$p < 0.05$, **$p < 0.01$

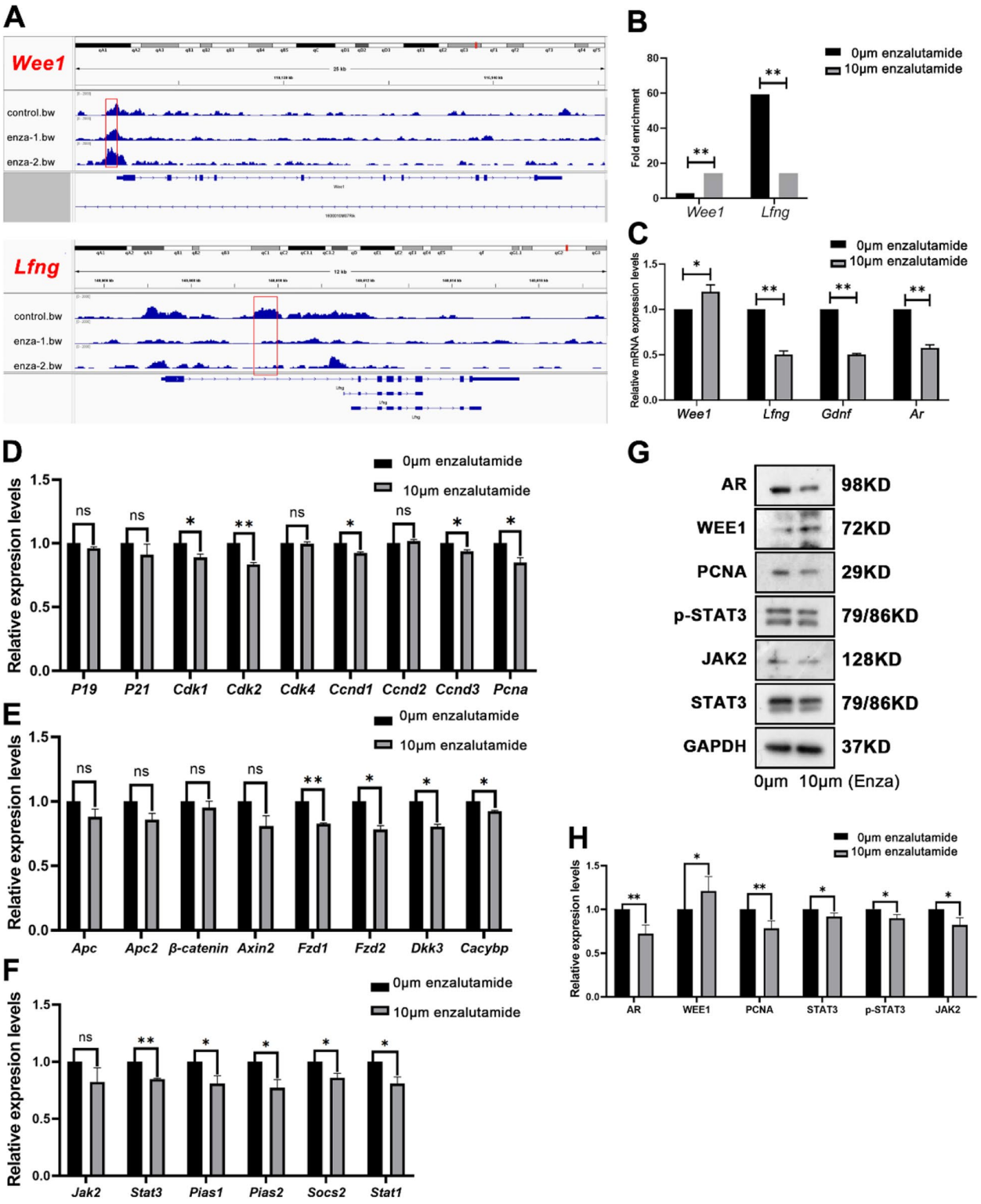

Fig. 4 (See legend on previous page.)

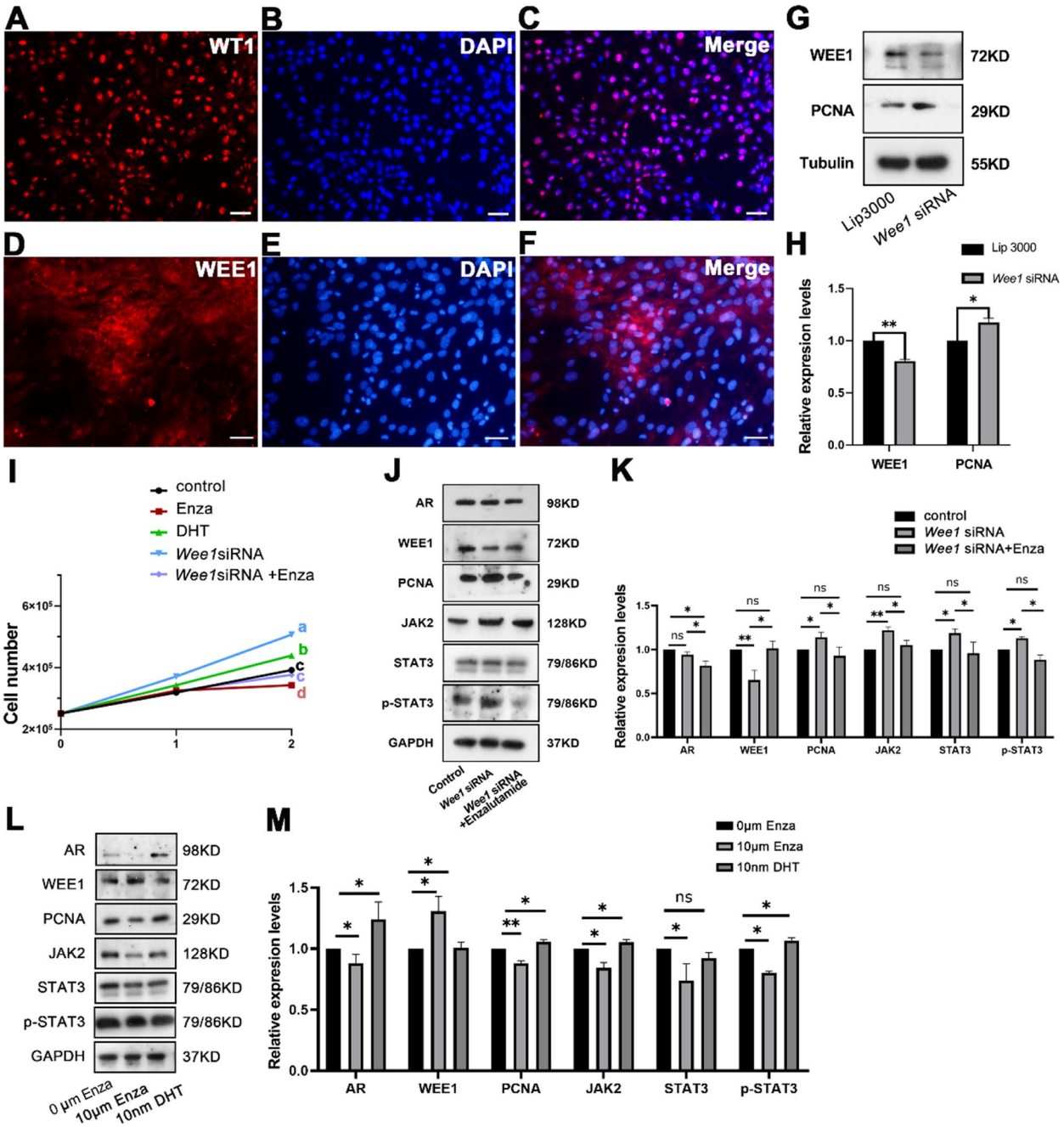

Fig. 5 Blockade of AR signaling inhibits proliferation of Sertoli cells via *Wee1*. **A**-**F** Immunostaining of WT1 and WEE1 in primary Sertoli cells (A. WT1, B. DAPI, C. merge, D. WEE1, E. DAPI, F. merge). **G** Expression levels of WEE1, PCNA, and β-tubulin in Sertoli cells treated with Lipofectamine 3000 or *Wee1* siRNA gauged by Western blotting. **H** Statistical analysis of the WB results of G. **I** Cell counts of Sertoli cells treated with vehicle, 10 μM enzalutamide, 10 nM DHT, *Wee1* siRNA, or *Wee1* siRNA + 10 μM enzalutamide across two passages. Statistical differences are denoted by a-d; groups labeled with different letters exhibit significant differences. **J** Western blot analysis of AR, WEE1, PCNA, JAK2, STAT3, and phosphorylated STAT3 in Sertoli cells treated with vehicle, Wee1 siRNA, or Wee1 siRNA + 10 μM enzalutamide. **K** Statistical analysis of Western blot results. **L** Expression of AR, WEE1, PCNA, JAK2, STAT3, and phosphorylated STAT3 in Sertoli cells treated with vehicle, 10 μM enzalutamide, or 10 nM DHT as determined by Western blotting. **M** Statistical analysis of these Western blot results. Results are presented as mean ± SD, with significance levels indicated by n.s. $p > 0.05$, *$p < 0.05$, **$p < 0.01$. Scale bar = 20 μm

an in-depth investigation of the mechanisms by which WEE1 regulates this process. Initially, we assessed WEE1 expression in Sertoli cells (Fig. 5A-F). Prior Western Blot analyses demonstrated that treatment with enzalutamide significantly elevated WEE1 expression levels (Fig. 4G-H). Subsequently, *Wee1* was silenced in Sertoli cells isolated from 7-day-old mouse testes, revealing that WEE1 knockdown resulted in an increased expression of PCNA (Fig. 5G, H). We further evaluated Sertoli cell proliferation by enumerating cell numbers over three generations, considering that primary Sertoli cells can be cultured in vitro for only 3–4 generations. Comparisons were made across different treatment groups: control, 10 μM enzalutamide, 10 nM dihydrotestosterone (DHT), *Wee1* knockdown, and combined *Wee1* knockdown with 10 μM enzalutamide treatment (Fig. 5I). The results indicated a decrease in cell numbers in the enzalutamide-treated group compared to the control, whereas an increase in cell numbers was observed in the 10 nM DHT-treated and *Wee1* knockdown groups. Notably, the simultaneous *Wee1* knockdown and enzalutamide treatment group exhibited cell numbers comparable to the control group. These data suggest that AR inhibition in Sertoli cells results in upregulated WEE1 expression, which, in turn, impedes Sertoli cell proliferation.

Moreover, our previous findings indicated alterations not only in WEE1 levels but also in the JAK/STAT signaling pathway after androgen blockage, implying that proliferation inhibition by WEE1 may work through JAK-STAT signaling pathway. To further test this, we examined JAK2 and STAT3 expression levels following *Wee1* knockdown and subsequent enzalutamide treatment, which could re-activate WEE1 expression after *Wee1*-knockdown. We observed that *Wee1* knockdown significantly increased JAK2 and STAT3 expression levels and STAT3 phosphorylation level, while enzalutamide treatment following *Wee1* knockdown suppressed JAK2 and STAT3 expression and reduced STAT3 phosphorylation levels compared to the *Wee1* knockdown group (Fig. 5J-K). This suggests that enzalutamide treatment can negate the effects of *Wee1* siRNA, reactivating WEE1 expression and function, thereby inhibiting the JAK-STAT signaling pathway. These findings propose that AR signaling in Sertoli cells may influence the JAK-STAT signaling pathway accompanied with regulation of WEE1, thus modulating neonatal Sertoli cell proliferation.

To validate this hypothesis, we treated Sertoli cells isolated from 7-day-old mouse testes with enzalutamide and DHT. The results mirrored those observed in the *Wee1* knockdown and activation experiments; androgen levels were negatively correlated with WEE1 expression and positively correlated with the activity of the cell proliferation-related gene PCNA and the key genes JAK2 and STAT3 in the JAK-STAT pathway (Fig. 5L-M). These findings elucidate that androgen signaling in Sertoli cells regulates neonatal Sertoli cell proliferation via the WEE1 and JAK-STAT pathways.

Blockage of AR signaling pathway inhibited production of GDNF in Sertoli cells via Notch signaling pathway

The aforementioned findings suggest that androgen blockade not only impacts Sertoli cell proliferation but also their secretion of GDNF. Prior studies have demonstrated that GDNF production in Sertoli cells is regulated by the Notch signaling pathway [21]. Moreover, the activity of NOTCH is modulated by LFNG modifications. LFNG acts as an antagonist to the Notch signaling pathway by enhancing the Notch receptor's affinity for DLL (a Notch inhibitory ligand) and preventing JAG (a Notch activation ligand) from binding to Notch1, thereby suppressing the Notch signaling pathway [32]. Combined with our earlier observations, we hypothesize that androgen blockade diminishes AR's binding affinity to the *Lfng* gene, which leads to downregulation of LFNG expression and subsequently reduces N-acetylglucosamine modification on NOTCH. Consequently, there is a diminished binding capability to DLL, resulting in the activation of the Notch signaling pathway and suppression of GDNF expression.

To validate this hypothesis, we analyzed the expression changes of components within the Notch signaling pathway. Results indicated that post-androgen blockade, there was downregulation in the expression of *Notch1*, *Notch2*, *Notch4*, and the Notch activator ligand *Jag2*. Additionally, there was a significant decrease in the Notch inhibitor ligand *Dll3*. Crucially, the expression of two key transcriptional repressors of the Notch signaling pathway, *Hes1* and *Hey1*, was markedly decreased, suggesting an overall activation of the Notch signaling pathway (Fig. 6A). To substantiate these findings further, we conducted Western blot analysis to monitor GDNF protein levels and observed a significant reduction in GDNF expression following androgen blockade, aligning with our predictions (Fig. 6B-C).

In conclusion, integrating our study's findings, we outline the potential molecular mechanism by which androgens modulate the proliferation and function of neonatal Sertoli cells. Under normal androgen levels, AR binding to the *Lfng* gene fosters LFNG expression, thereby regulating NOTCH glycosylation, suppressing the Notch signaling pathway, and promoting GDNF expression, which is essential for SSC self-renewal. Concurrently, AR's relatively weak binding to *Wee1* allows the CDK/cyclin complex to govern Sertoli cell mitosis. However, upon androgen inhibition, Sertoli cells exhibit a sensitive response to androgen level changes via AR. This results

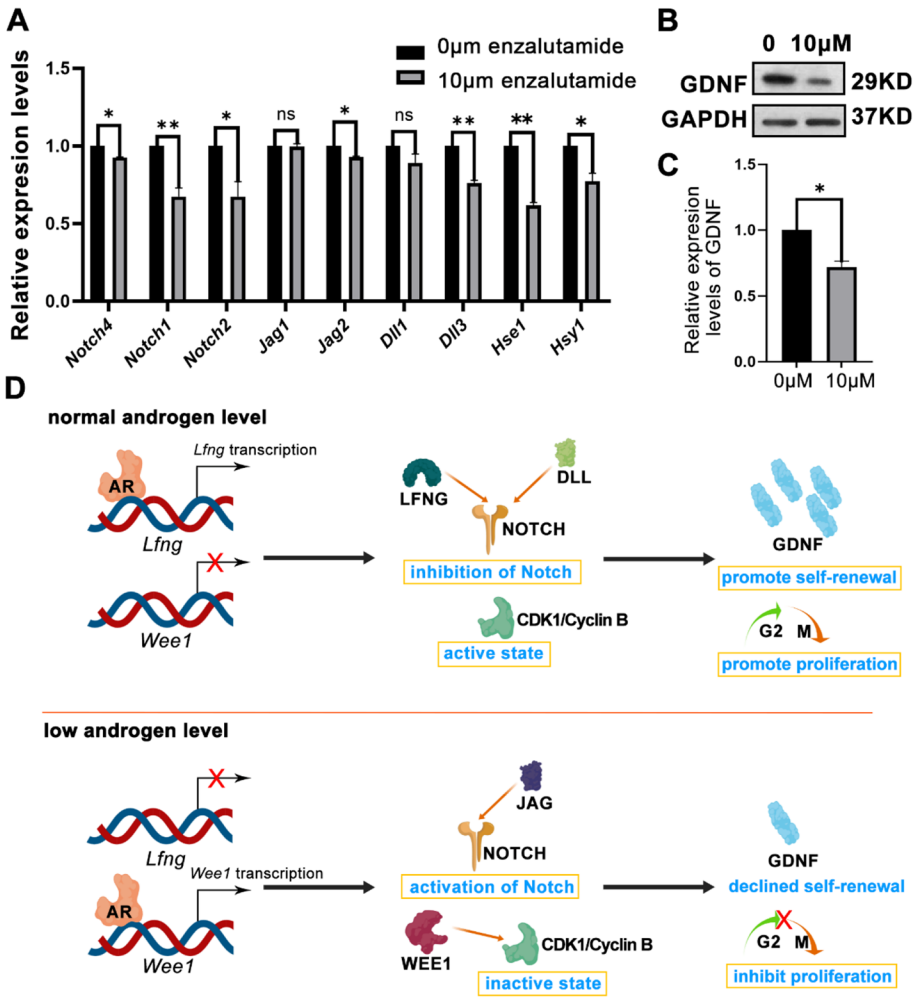

Fig. 6 Blockade of AR signaling inhibits GDNF production in Sertoli cells via Notch signaling. **A** RT-PCR analysis of proliferation-associated genes (*Notch4*, *Notch1*, *Notch2*, *Jag2*, *Jag1*, *Dll1*, *Dll3*, *Hse1*, *Hsy1*) in Sertoli cells treated with DMSO or 10 μM enzalutamide. **B** Western blot analysis of GDNF and GAPDH in Sertoli cells treated with DMSO or 10 μM enzalutamide. **C** Statistical representation of the Western blot results. Results are presented as mean ± SD, with significance denoted by n.s. $p > 0.05$, *$p < 0.05$, **$p < 0.01$. **D** Summary of the regulatory effects of androgen levels on Sertoli cell proliferation and GDNF production as derived from this and previously published studies

in altered AR targets, including diminished AR binding to the *Lfng* gene, reducing LFNG expression, activating the Notch signaling pathway, and consequently lowering GDNF expression. Moreover, there is an enhanced AR binding to the *Wee1* gene, increasing its expression. As a proliferation inhibitor, WEE1 impedes CDK1/Cyclin B activity, leading to inhibited cell proliferation (Fig. 6D).

Discussion

Sertoli cells play an essential role in the testes by interacting with germ cells and regulating their functions, including migration, proliferation, and differentiation [33, 34]. Furthermore, they interact with other somatic cells within the testes to regulate their biological functions, for example immune homeostatsis [35] and Leydig cell steroidogenesis [36]. Androgens secreted by Leydig cells regulate the function of Sertoli cells, while Sertoli cells can also influence the development and function of Leydig cells. Androgen levels are critical for testicular development and function. This study focuses on their impact during the early stages of testicular development, particularly during the first proliferation period of Sertoli cells. In mice, two waves of Sertoli cell proliferation occur during the neonatal period and puberty, respectively [8]. Although androgens play a vital role in the development and function of Sertoli cells, the underlying molecular mechanisms remain partially understood. The quantity of Sertoli cells determines the volume and normal function of the testes [37]. Once Sertoli cells complete their two proliferation

phases, they lose the ability to divide [8]. Therefore, any impact on their proliferation can severely affect testicular function, particularly spermatogenesis.

Through ChIP-seq experiments, we found that upon AR antagonism, the binding of AR to the promoter region of *Wee1* increased, consequently activating the expression of the proliferation-inhibiting gene *Wee1*, which obstructed Sertoli cell proliferation. AR, as a significant transcription factor, binds or dissociates from target genes in a dynamic equilibrium. Enzalutamide partially inhibits AR activity, simulating a condition of low androgen levels. In this scenario, we observed altered AR target gene binding, indicating that fluctuations in androgen levels trigger changes in the expression profiles of Sertoli cells. One significant impact is on the proliferation capacity of Sertoli cells. Hence, we hypothesize that androgen levels are essential for neonatal Sertoli cell proliferation. Under low androgen levels, AR binds to and activates *Wee1* expression, leading to an inhibitory effect on cell proliferation in neonatal Sertoli cells.

It's interesting that a reduction in androgen levels inhibits Sertoli cell proliferation. We propose that androgen levels are vital signals for testicular development and functions since androgen simultaneously acts on multiple cell types, including Sertoli cells, myoid cells, and even precursor spermatogonial cells within 0–2 days post-birth [7]. AR affects multiple target genes involved in DNA repair, apoptosis, cell cycle, and differentiation. Therefore, AR may sensitively respond to androgen levels. When androgen levels decrease, cells may transition from the proliferative developmental stage to a quiescent phase. *Wee1* might be a critical switch regulating this cellular state transition. Notably, when we compared the proliferation of primary Sertoli cells under normal culture conditions, DHT treatment, enzalutamide treatment, *Wee1* siRNA treatment, and *Wee1* siRNA + enzalutamide treatment, we observed that cells treated with Wee1 siRNA had significantly higher proliferation rates than other groups, even higher than the DHT-treated group. This suggests that *Wee1* is a core gene inhibiting Sertoli cell proliferation. Overexpression of *Wee1* led to increased cell size at division, and excessive overexpression resulted in cell death [38]. However, in our results, blocking androgen receptor activity did not cause significant cell enlargement or death, possibly because the rise in *Wee1* expression due to decreased androgen levels was only about 60% (Table S4), far less than the multiple times increase observed with overexpression. This result suggests that hormone level fluctuations have a relatively moderate impact on downstream target genes, primarily affecting the proliferation capacity of Sertoli cells and, consequently, the developmental potential and function of the testes.

In addition to affecting proliferation, decreased androgen levels directly cause reduced GDNF production, impacting the self-renewal of spermatogonial stem cells. Our results show that decreased androgen levels weaken AR binding to *Lfng* and significantly lower its expression, affecting modifications to the Notch signaling pathway. The Notch signaling pathway is a highly conserved cell signaling mechanism widely involved in various biological processes, including cell differentiation, proliferation, and apoptosis [39]. Our KEGG and GSEA results also showed that genes related to apoptosis, proliferation, and energy metabolism are affected. However, it remains unclear if these changes are mediated through the Notch signaling pathway. The activity of Notch is dynamically regulated by two types of ligands, DLL and JAG [24]. Both spermatogonial cells and adjacent Sertoli cells can release Notch ligands to regulate Sertoli cells. In this process, Notch modifications play a critical role. We hypothesize that LFNG enhances DLL binding and reduces JAG binding by modifying the NOTCH protein. Since JAG is a critical ligand activating NOTCH in Sertoli cells, high LFNG expression, increased NOTCH modification, and decreased JAG binding lead to decreased Notch activation and increased GDNF expression. This regulatory model hints at the complex regulatory mechanisms within the testicular tissue, suggesting that different cell types might secrete various factors through paracrine actions to drive different functional outcomes. However, these conclusions are derived from existing literature and cell experiments, requiring more in-depth mechanistic studies, especially in vivo models, for verification.

Conclusion

Our findings emphasize the pivotal role of androgen levels in the proliferation and function of testicular Sertoli cells. We have demonstrated that androgen receptor activity significantly influences the expression of crucial genes like *Wee1*, affecting the proliferative capacity of Sertoli cells. The interplay between androgen levels and other signaling pathways, particularly the Notch pathway, further complicates the regulatory landscape of testicular development and function. Future research should focus on in vivo models to elucidate the exact mechanisms by which androgen fluctuations regulate Sertoli cell proliferation and other testicular functions. Understanding these intricate interactions will provide insights into potential therapeutic targets for male fertility disorders and pave the way for advanced reproductive technologies.

Abbreviations

AR	Androgen receptor
CDKs	Cyclin-dependent kinases
DEGs	Differential expressed genes
FBS	Fetal bovine serum
GDNF	Glial cell line-derived neurotrophic factor
SSCs	Spermatogonial stem cells
DHT	Dihydrotestosterone
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
IGV	Integrative Genomics Viewer

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12964-024-01875-5.

Additional file 1: Table S1. The Information of primers used in this study.

Additional file 2: Table S2. The Information of antibodies used in this study.

Additional file 3: Table S3. Peakcalling result.

Additional file 4: Table S4. Differential expressed genes of RNA-seq results.

Additional file 5: Table S5. The integration analysis of ChIP-seq and RNA-seq results.

Additional file 6.

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Authors' contributions
W.H.Z. contributed to data collection, data analysis, and securing funding support. R.H.T. prepared cell samples, collected and assembled data, and participated in data analysis and interpretation. X.M.L., J.W., and Y.Q.W. contributed to data analysis, validation, and visualization. Z.P.L. assisted with data analysis and manuscript revision. X.D.Z. participated in data interpretation and visualization. L.T. conceived the study, contributed to data analysis, and revised the manuscript. K.Z. secured funding support, conceived the study, designed the experiments, wrote, revised, and finalized the manuscript.

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Data availability
The data that supports the findings of this study are available in the method part and supplemental materials. To download the original data of ChIP-seq and RNA-seq, please visit the GEO database website at https://www.ncbi.nlm.nih.gov/gds/ and use the code:PRJNA1140838.

Declarations

Ethics approval and consent to participate
All experiments in this study have been approved by the ethical committee of Nanjing Agricultural University.

Consent for publication
All authors agree to publish this manuscript.

Competing interests
The authors declare no competing interests.

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