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CircRNA profiling reveals circSMC1B that promotes bovine male germline stem cells proliferation and apoptosis via sponging let-7i

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Abstract

Background Despite significant advancements in artificial insemination techniques, male reproduction continues to pose a considerable challenge in cattle breeding. Circular RNAs (circRNAs), a class of non-coding RNAs (ncRNAs), play a crucial role in regulating testis growth and spermatogenesis. Therefore, it is essential to comprehend the involvement of circRNAs in bull reproduction for livestock production. However, the identification of differentially expressed circRNAs during testis development and their underlying mechanisms remains largely unknown.

Results In this study, RNA-seq was employed to investigate the expression of circRNAs in neonatal and sexually mature bovine testes. We identified 28,065 candidate circRNAs, of which 987 circRNAs showed differential expression between the two stages (*P*-adjust < 0.05). Notably, circSMC1B was significantly up-regulated in sexually mature testis. Overexpression of circSMC1B promoted the proliferation and apoptosis of bovine male germline stem cells (mGSCs). Further analysis revealed that circSMC1B acts as a molecular sponge for let-7i, while High mobility group AT-hook 1/Nuclear receptor subfamily 6 group A member 1 (*HMGA1/NR6 A1*) were identified as direct targets of let-7i. Furthermore, circSMC1B levels exhibited a significant positive correlation with *HMGA1/NR6A1* mRNA expression in bovine mGSCs, highlighting the critical role of competing endogenous RNA (ceRNA) mechanisms.

Conclusion Our research elucidates that circSMC1B, through let-7i binding, promotes bovine mGSCs proliferation and apoptosis by targeting *HMGA1/NR6A1*. These findings provide valuable resources for studying the functional aspects of circRNAs in testis development and enhance our understanding of the biological function of circSMC1B in promoting bull spermatogenesis.

Keywords CircRNA, Let-7i, Bull spermatogenesis

Introduction

Circular RNAs (circRNAs) are a class of non-coding RNAs (ncRNAs) characterized by a covalently closed-loop structure. Despite the initial discovery of circRNA dating back to 1979 [1], in-depth studies were limited until recently due to technical constraints. These molecules are generated through back-splicing, a process in which splicing proceeds in the reverse direction during the maturation of linear pre-mRNA [2]. Due to their circular structure, circRNAs lack free 5'and 3'ends, making them more stable than linear RNAs and resistant to degradation by Ribonuclease R (RNase R) [3]. CircRNAs

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can originate from exons or introns and are classified into three main subtypes. They exert their functions through various mechanisms, including acting as miRNA sponges or competing endogenous RNAs (ceRNAs) to upregulate the expression of miRNA target genes, thereby contributing to post-transcriptional gene regulation and influencing a wide range of biological processes and diseases [4, 5]. Additionally, they can serve as protein sponges or decoys, influencing protein function [6]. Growing evidence highlights their potential as tissue-specific and highly stable biomarkers in various diseases due to their abundant presence, stability, and evolutionary conservation across species [7]. Consequently, circRNA research has garnered significant attention in biological sciences.

In recent years, circRNAs have emerged as a topic of interest in male reproduction research. Testicular circRNAs were initially identified in mice from the *Sry* gene (Sex-determining Region Y) [8]. CircSry (Sry CircRNA) regulates gene expression as a miR-138 sponge [9]. Dong and colleagues employed next-generation deep sequencing to identify over 15,000 circRNAs in human testes and seminal plasma, of which 10,792 (67%) were novel. They also identified 1,017 circRNA host genes related to spermatogenesis or sperm motility and fertilization processes [10]. Their findings demonstrated the stability of circRNAs and suggested their potential use as non-invasive biomarkers for male infertility. In mice, tissue-specific circRNAs were found in both brains and testes, with the number of tissue-specific circRNAs ranking second only to those in the brain [11]. Zhou et al. discovered that circRNA expression in testes significantly increases with sexual maturity and decreases with aging, indicating specific physiological activities of circRNAs rather than mere by-products of RNA processing. They also proposed that circRNAs can serve as biomarkers of reproductive aging [12].

Several studies have focused on the role of circRNAs in human male infertility, especially in cases of azoospermia, asthenozoospermia, and oligoasthenozoospermia, using samples from testes, semen, and blood. These studies have identified circRNAs as potential biomarkers, therapeutic targets, and candidates for pharmaceutical intervention in male reproduction research [13]. Ji et al. constructed networks comprising differentially expressed circRNAs (hsa_circPDE3B_001, hsa_circZHX3_008, hsa_circFAM114 A2_005) that are implicated in the Wnt signaling pathway and play a crucial role in the pathogenesis of non-obstructive azoospermia (NOA). Furthermore, they demonstrated the utility of seminal plasma (SP) circRNAs as biomarkers for predicting the outcome of microdissection testicular sperm extraction (micro-TESE) in NOA patients [14]. Two studies reported that some differentially expressed circRNAs (circUSP54) are primarily associated with mitochondrial physiology and energy production [15, 16]. The latest research reveals that the Rsrc1-161aa protein encoded by circRsrc1 regulates mitochondrial ribosome assembly and translation during spermatogenesis, affecting male fertility [17]. However, despite limited studies exploring their expression patterns and potential roles in fertility-related processes, the investigation of circRNAs in this field is still in its early stages.

In livestock, our study first identified 2,225 up-regulated and 2,023 down-regulated circRNAs in the testes of neonatal (1 week old) and adult (4 years old) Qinchuan cattle. These circRNAs were associated with male reproductive and spermatogenesis-related genes [18]. Li et al. demonstrated circRNA expression at different reproductive stages in Tibetan sheep testes using high-throughput RNA sequencing [19]. Zhang and colleagues identified 2,326 differentially expressed circRNAs in the testes of adult boars and piglets, with some associated with spermatogenesis (circRNA 10979) and germ cell development (circRNA 18456). CircRNAs 6682, 10187, 18456, and 10979 were upregulated in adult boar testes, suggesting their potential use as indicators of sexual maturation in pigs [20]. Khan et al. identified 17,013 circRNAs in testicular samples collected from six 3-year and 3-month-old Wandong bulls, among which 681 were differentially expressed [21]. Additionally, 277 circRNAs were identified as playing pivotal roles in yak testicular development and spermatogenesis [22], and 137 differentially expressed circRNAs were identified in the epididymis of yaks and cattleyaks [23]. In conclusion, livestock circRNA function research heavily relies on bioinformatics. However, our understanding of circRNA expression profiles in cattle testes is limited, and there has been no study on the effects of circRNA on the development of bovine testicular cells.

Male germline stem cells (mGSCs) play a crucial role in spermatogenesis, the process by which sperm cells are produced in the testes [24]. Recent studies have highlighted that circRNAs have been shown to influence signaling pathways that are critical for stem cell maintenance and fate determination [25, 26]. Their stable expression profiles and tissue-specific regulation make circRNAs potential biomarkers for assessing mGSC functionality. Understanding the interplay between circRNAs and mGSCs could provide valuable insights into male fertility.

To assess the potential impact of circRNAs on the development of bovine testes from birth to sexual maturity, we conducted a comprehensive screening of the circRNA expression profile in Angus bovine testes at neonatal and sexual mature stages using Ribo-Zero RNA-seq. Furthermore, we performed GO and KEGG enrichment analyses on the host genes of differentially

expressed circRNAs to gain insights into their functions in testicular development and spermatogenesis. We focused on circSMC1B, which showed differential and strong expression in two developmental stages. We further investigated its role in the proliferation and apoptosis of mGSCs. The findings of this study contribute to the existing genetic resources, furthering our understanding of bovine sexual maturity processes, and shedding new light on the function of circRNAs in regulating the proliferation and apoptosis of bovine mGSCs.

Materials and methods

Sample preparation

Six whole testes (3 days after birth [Neonatal, N] and 13 months [Mature, M]) from Angus bulls were collected from Shaanxi Kingbull Livestock Co., Ltd. (Baoji, China). A volume of 3 ml of 2% Lignocaine was injected as a local anesthetic into the contralateral pole of each testicle in Angus bulls. Subsequent to the standard surgery skin preparation, the testicles were carefully removed. After the incision wounds of these animals were disinfected with a 20% chlorhexidine solution, these animals were released, fed and cared separately. Their testicular samples were dissected, and then immediately frozen in liquid nitrogen. The information on testicular samples was provided by Gao et al. [27]. The raw data of cDNA libraries from the testes of six Angus bulls were downloaded from the Sequence Read Archive database (SRP148084).

CircRNA identification and differential expression analysis

Firstly, raw data were checked using FastQC (v0.11.9). Adapters were removed using Trimmomatic (v0.39) [28], and the low-quality reads were filtered out with default parameters. Then, paired-end clean reads were aligned to the reference genome (B. Taurus UMD3.1) using HISAT2 (v2.0.3) [29] and Bowtie2 (v2.2.8) [30]. Find_circ (v1.0) was used to identify circRNA [5]. CircRNA expression in each sample was counted, and DESeq2 (v1.34.0) [31] based on a negative binomial distribution was used for the differential expression analysis of circRNA (*P*-adjust < 0.05, |log2 FoldChange|> 1).

GO terms and KEGG pathway enrichment

The GOseq R package was used to perform Gene Ontology (GO) enrichment analysis of the host genes of differentially expressed circRNAs, with adjustments made to correct for gene length bias [32]. GO terms with a corrected *P*-value less than 0.05 were considered significantly enriched. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the host gene of differentially expressed circRNA was conducted using the KOBAS software [33]. KEGG pathways with a *P*-value less than 0.05 were considered significantly enriched.

Cell culture

Human embryonic kidney 293 T (HEK293 T) cells (ATCC) were cultured in high-glucose DMEM (HyClone, Logan, UT, USA) with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA). Bovine mGSCs were derived from an immortalized cell line [34], which was < P15 with male germline stem cell properties after immortalization. The culture medium of mGSCs consisted of DMEM/F12 supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine (Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen). Cells were seeded in a 60-mm plate (2×10^6 cells/well) with 4 mL medium in 5% CO_2 at 37 °C. Cells were plated at 5×10^5 cells/well in 6-well plates or 1×10^4 cells/well in 96-well plates (NEST, Wuxi, P.R. China). Cells were treated with let-7i mimic (2 mg/mL, RuiboBio, Guangzhou, China), pCD2.1-circSMC1B (2 mg/mL) or let-7i mimic + pCD2.1-circSMC1B at 80% confluence.

Plasmid construction

The full length of circSMC1B (Fig. S1) was cloned into pCD2.1-ciR (Geneseed, Guangzhou, China), which contained a front and back circular frame, while the mock vector with no circSMC1B sequence served as a control. We amplified the full length of circSMC1B by psiCHECK2-circSMC1B-F and psiCHECK2-circSMC1B-R primers. We inserted it into the psi-CHECK-2 vector (Promega, Madison, WI, USA) at the 3'end of the Renilla gene using restriction enzymes XhoI and NotI (NEB, Beijing, China) and T4 DNA ligase (Takara, Dalian, China). The mutant psiCHECK2-circSMC1B-3'UTR-Mut was obtained by mutating sequences complementary to the seed region of the let-7i using a mutagenic primer. Similarly, we generated the vectors of psiCHECK-HMGA1/NR6A1-W/Mut. A let-7i sensor was created by inserting two consecutive let- 7i complementary sequences into the psiCHECK-2 vector. Primer sequences are listed in Table S1.

RNA extraction and quantitative real-time PCR

All experimental procedures adhered to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [35]. Total RNA was extracted using RNAiso Plus (TaKaRa, China) following the manufacturer's instructions. The RNA concentration was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific), and 500 ng was used as starting material for cDNA synthesis,

applying the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, China) according to the provided protocol. For quantitative RT-PCR analysis, the circRNA primers and other primers were designed using Primer Premier 5.0 software (Table S2). The reference genes *β-actin* were utilized as internal controls to normalize gene expression levels. Quantitative RT-PCR was carried out using AceQ SYBR Green PCR Master Mix (Vazyme, Nanjing, China) on a Bio-Rad CFX96 instrument. The PCR program included an initial denaturation step at 95 °C for 5 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s, and a melt cycle from 60 to 95 °C in 0.5-s increments. The efficiencies of the RT-qPCR primers were calculated using a cDNA template dilution series, and amplification specificity was verified using melting-curve analysis. Each sample was analyzed with three biological replicates, and relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method [36].

CCK-8 and EdU assays

The CCK-8 (Multisciences, Hangzhou, China) and EdU assays (RiboBio, Guangzhou, China) were used to examine cell proliferation. For the CCK-8 assay, the cells were plated into 96-well culture plates at a density of 5×10^3 cells/well in 100 μL of culture medium/well. 10 μL of CCK-8 reagent was added to each well and incubated for 2 h after transfection 24 h. Then, the optical density (OD) value was measured at a wavelength of 450 nm. The Cell-Light™ EdU kit was used to detect cell proliferation status, and the number of EdU-positive cells was calculated under the fluorescence microscope (AMG EVOS, Seattle, WA).

Cell cycle assay

A cell cycle testing kit (Multisciences, Hangzhou, China) and flow cytometry (FACS Canto II, BD Biosciences, USA) were used to analyze the cell cycle according to the manufacturer's instructions. Three replicates were used in each treatment group.

Hoechst 33,342/PI dual staining assay

Hoechst 33,342 and propidium iodide (PI) double staining (Solarbio, Beijing, China) was used to test cell apoptosis. After 24 h treatment, mGSCs were treated with Hoechst 33,342 for 15 min and PI for 10 min at room temperature. The fluorescence signal was detected by a fluorescence microscope (DM5000B, Leica Microsystems, Germany).

Luciferase activity assay

The psiCHECK-circSMC1B-Wild or psiCHECK-circSMC1B-Mut plasmid and the let-7i mimic were transfected into HEK293 T when the cell confluence reached approximately 80%. The cells were seeded into 24-well plates in triplicate and co-transfected with a wild-type or mutant construct of High mobility group AT-hook 1/Nuclear receptor subfamily 6 group A member 1 (*HMGA1* and *NR6A1*) with and without let-7i. After 48 h, the Dual-Luciferase reporter assay system kit (Promega, USA) was used to analyze luciferase activities according to the manufacturer's instructions.

Statistical analysis

The raw data were compiled using MS Excel 2021 software (Microsoft, Redmond, WA, USA), and one-way analysis of variance (ANOVA) was performed with SPSS 19.0 (IBM, Armonk, NY, USA). Statistical analysis was performed to assess significant differences in relative expression between the neonatal and mature groups, and between the negative control and experimental (circSMC1B or let-7i) groups. The confidence interval was set to 95%, and a *P*-value threshold of <0.05 was chosen to indicate statistical significance. Additionally, GraphPad Prism (version 9.5, GraphPad Software Inc., MA, USA) was utilized for graphic illustrations to enhance the presentation of the experimental results. All data are presented as mean ± standard error (SE). Statistical significance was defined as * $P<0.05$, ** $P<0.01$.

Results

CircRNA expression patterns in mature and neonatal bovine testicular tissue

We screened RNAs in neonatal and mature bull testis using RNA-seq, obtaining 89-106 (94.62%-95.19%) and 86-91 (94.17%- 95.19%) million mapped clean reads from the neonatal and sexually mature bull testes libraries, respectively (Table 1). Additionally, 4.5-5.9 (4.81%-5.38%) and 4.5-5.5 (4.81%-5.83%) million unmapped clean reads from neonatal and sexually mature bull testis libraries were used to predict putative circRNA. A total of 28,065 candidate circRNAs were detected (Table S3), of which 12,428 and 8,179 circRNAs were exclusively detected in neonatal and mature testes, respectively (Fig. 1a). Then, we classified the sources of candidate circRNAs. The majority were intron-derived sequences, followed by exon-derived sequences, while intergenic splice sequences were the least abundant (Fig. 1b).

Differentially expressed circRNAs in bovine testicular tissues

Differential expression analysis of circRNAs revealed that 987 circRNAs were differentially expressed (DE) in testicular tissues of Angus bulls at different developmental stages (Tables S4, S5). Specifically, 710 circRNAs were upregulated, while 277 were downregulated when comparing neonatal tissues to mature tissues (*P*-adjust

Table 1 Summary of reads mapping to the cattle reference genome

Sample	Raw reads	Clean reads	Mapped reads	Rate	Unmapped reads	Rate
M1	94,866,620	91,525,762	86,955,668	95.01%	4,570,094	4.99%
M2	99,355,310	96,639,934	91,991,361	95.19%	4,648,573	4.81%
M3	98,676,988	95,105,056	89,562,538	94.17%	5,542,518	5.83%
N1	97,715,528	94,414,154	89,337,136	94.62%	5,077,018	5.38%
N2	96,661,530	93,742,744	89,229,051	95.19%	4,513,693	4.81%
N3	115,633,006	112,007,198	106,066,039	94.70%	5,941,159	5.30%

M1-M3, means samples aged 13 months; N1-N3, means samples taken 3 days after birth

< 0.05) (Fig. 1c). To further investigate the potential roles of these circRNAs, we created a clustered heatmap of the DE circRNAs (*P*-adjust < 0.05, Fig. 1d).

Enrichment analysis of DE circRNAs

GO enrichment analysis indicated that the host genes of DE circRNAs were significantly enriched with 36 GO terms (corrected *P*-value < 0.05), including 22 biological processes and 14 molecular functions (Fig. 1f, Table S6). The host genes were particularly enriched in biological processes such as sexual reproduction, gamete generation, germ cell development, spermatid development, spermatogenesis, male gamete generation, spermatid differentiation, etc. In terms of molecular functions, the circRNA host genes were enriched in activities such as adenyl ribonucleotide binding, adenyl nucleotide binding, purine ribonucleoside triphosphate binding, and ATP binding. KEGG enrichment analysis showed that the DE circRNA host genes were significantly enriched in pathways such as lysine degradation, focal adhesion, purine metabolism, progesterone-mediated oocyte maturation, adherens junction, fanconi anaemia pathway, cell cycle, ubiquinone and other terpenoid-quinone biosynthesis (*P*-value < 0.05). The top 20 pathways are illustrated in Fig. 1e and Table S7.

Verification of differentially expressed circRNA

To confirm the accuracy of high-throughput sequencing, 10 reproduction-related circRNAs were randomly selected. RT-PCR was performed to detect the back-splicing junction of these circRNAs. The sequencing results of PCR products, shown in Fig. S2, confirmed the existence of these circRNAs. Six pairs of circRNA primers yielded a single-peaked melting curve in RT-qPCR. The expression pattern of circRNAs in Angus bull testes at two developmental stages was detected by using RT-qPCR. The results validated the accuracy of the sequencing data by comparing the expression levels of circRNAs detected by RT-qPCR with those obtained from sequencing (Fig. 2a). These circRNAs can be utilized to investigate the mechanisms underlying testis development and spermatogenesis.

Expression of circSMC1B in Angus bull

To explore the function of candidate circRNAs, we compared our findings with circRNA data from Qinchuan bulls [18]. The circRNA bta_circ_0021665 was found to be differentially expressed in Qinchuan bulls at neonatal and adult stages, suggesting its significant role in male reproduction. For further investigation, we selected bta_circ_0021665 and renamed circSMC1B based on the host gene name. Subsequently, the full-length sequence of circSMC1B in Angus cattle testis was identified by Sanger sequencing (Fig. 2b). The expression pattern of circRNA in Angus bull tissue showed that circSMC1B was most highly expressed in the testis (Fig. 2c). Additionally, we measured the expression of circSMC1B in calf Sertoli cells, Leydig cells, and mGSCs, finding that the expression level of circSMC1B was highest in mGSCs (Fig. 2d). This suggests that circSMC1B plays a more important regulatory role in mGSCs. Therefore, we selected mGSCs as model cells to explore the effect of circSMC1B on their function.

CircSMC1B promotes bovine mGSCs proliferation and apoptosis

CircSMC1B expression was significantly increased by transfecting pCD2.1-circSMC1B into mGSCs (Fig. 3a). Notably, the expression of SMC1B remained unchanged following overexpression of circSMC1B (Fig. 3b), indicating the high efficiency of cyclization by pCD2.1-circSMC1B. The CCK-8 assay demonstrated that overexpression of circSMC1B significantly increased mGSCs' proliferation (Fig. 3c). Additionally, pCD2.1-circSMC1B significantly upregulated *Cyclin D1* expression and downregulated *P21* expression at the mRNA level (Fig. 3d). EdU staining assays revealed that circSMC1B overexpression led to a significant increase in the number of EdU-positive cells, indicating a proliferative effect (Fig. 3e, f). Consistent with these findings, cell cycle analysis showed that circSMC1B significantly increased the number of mGSCs in both S-phase and G2-phase, while significantly decreasing the number of cells in the G1 phase compared to the control group (Fig. 3g-i). These

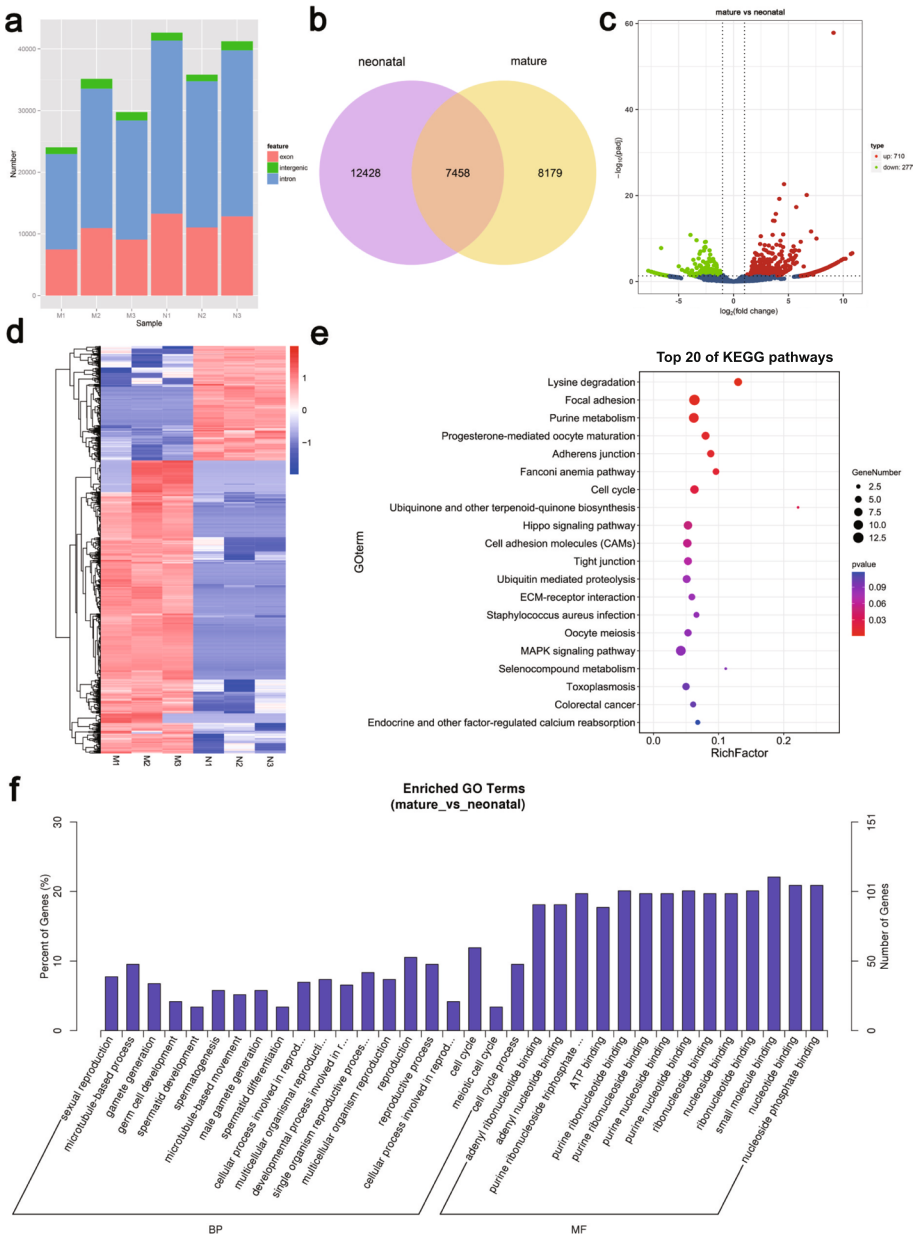

Fig.1 Identification and characterization of circular RNAs in Angus bull testes. **a** Origin of circRNAs described in this study in the bovine genome. **b** Venn diagram depicting different circRNAs uncovered in neonatal and mature bovine testis tissue. 28,065 candidate circRNAs were identified, with 12,428 and 8,179 specific to the neonatal and mature libraries, respectively. **c** Differential expression analysis using DESeq2. The volcano plot shows the correlation of abundances of individual circRNAs in the neonatal or maturation period (*P*-adjust < 0.05). **d** Clustered heatmap of differentially expressed circRNAs (*P*-adjust < 0.05). **e** KEGG pathway for significantly differentially expressed circRNAs (*P* < 0.05). **f** GO terms for significantly differentially expressed circRNAs (corrected *P*-value < 0.05)

results confirm that circSMC1B promotes the proliferation of bovine mGSCs.

To examine the role of circSMC1B in mGSCs apoptosis, the expression of apoptosis-related genes was detected by RT-qPCR following overexpression of circSMC1B. The expression levels of *Bcl-2*, *Bax* and *Caspase 9* were significantly increased, while the expression of *Caspase 3* was increased and the ratio of *Bcl-2/Bax* decreased compared with the control group (Fig. 3j). Flow cytometry analysis showed an increase in the number of double-stained Annexin V-FITC/PI cells (B2/B4) following circSMC1B overexpression (Fig. 3k-m). These results confirm that circSMC1B promotes proliferation and apoptosis in bovine mGSCs.

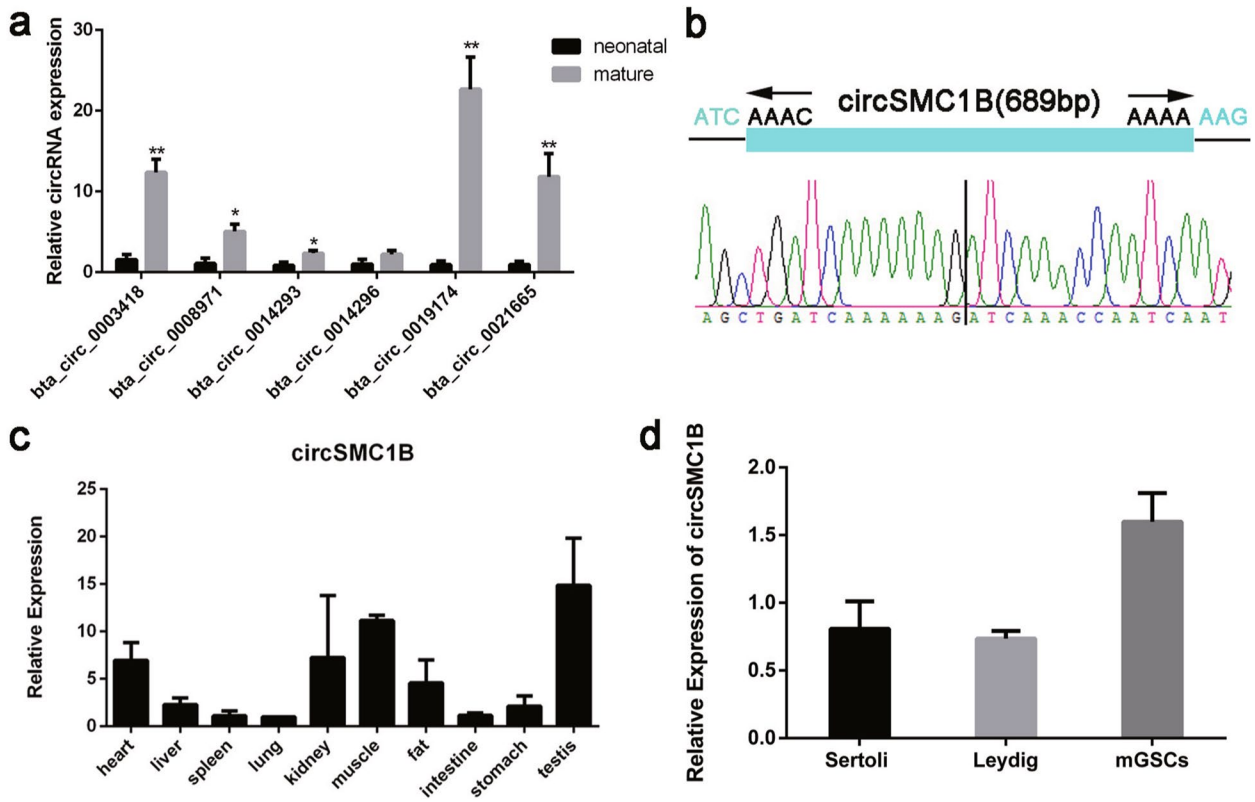

Fig. 2 The expression level of circSMC1B. **a** Validation of the expression of six circRNAs in three neonatal and mature bovine testis tissues using qPCR is shown. **b** Schematic view illustrating the design of primers for circSMC1B used in qPCR and Sanger sequencing of the junction of back-spliced products. **c** CircSMC1B is expressed in different tissues of Angus bulls at the neonatal stage. **d** The expression of circSMC1B in different cells of the bovine testis

let-7i bind to circSMC1B

Using the online software RNAhybrid, we predicted the binding sites of circSMC1B for miRNA and identified two binding sites for let-7i (Fig. 4a). The luciferase assay demonstrated that let-7i significantly inhibited the expression of Renilla luciferase (Rluc) when co-expressed with psi-check2-circSMC1B (Fig. 4c). Additionally, the let-7i sensor vector was constructed to further confirm the direct binding between circSMC1B and let-7i (Fig. 4b). The let-7i mimic significantly reduced the Rluc expression of the let-7i sensor, but the Rluc expression of the let-7i sensor was partially restored when overexpressed with circSMC1B (Fig. 4d). These results confirm that circSMC1B functions as a ceRNA for let-7i.

let-7i repress bovine mGSCs proliferation and apoptosis

The let-7i was revealed direct binding circSMC1B, to explore the effect of let-7i on bovine mGSCs proliferation and apoptosis, let-7i mimics were transfected into bovine mGSCs and then tested by flow cytometry, CCK-8, RT-qPCR, EdU assays. CCK-8 assays showed that let-7i can significantly reduce mGSCs viability after overexpressing let-7i (Fig. 5a). The let-7i significantly decreased the expression of *PCNA*, *CDK2*, and *CyclinD1* at the mRNA level (Fig. 5b). EdU staining assay showed that overexpression of let-7i significantly decreased the number of EdU-positive cells (Fig. 5c/d). Cell cycle analysis showed that let-7i significantly decreased the number of mGSCs in the S phase while significantly increasing the number of cells in the G1 phase (Fig. 5e).

To investigate the role of let-7i in mGSCs apoptosis, we detected apoptosis-related gene expression by RT-qPCR following let-7i overexpression. The expression levels of *Bax* and *Caspase 9* were significantly decreased, while the *Bcl-2/Bax* ratio was significantly increased compared with the control group (Fig. 5f). Flow cytometry analysis revealed that the number of double-stained Annexin V-FITC/PI cells (B2/B4) decreased following the overexpression of circSMC1B, in comparison to the control group (Fig. 5g/h). These results confirm that let-7i represses the proliferation and apoptosis of bovine mGSCs.

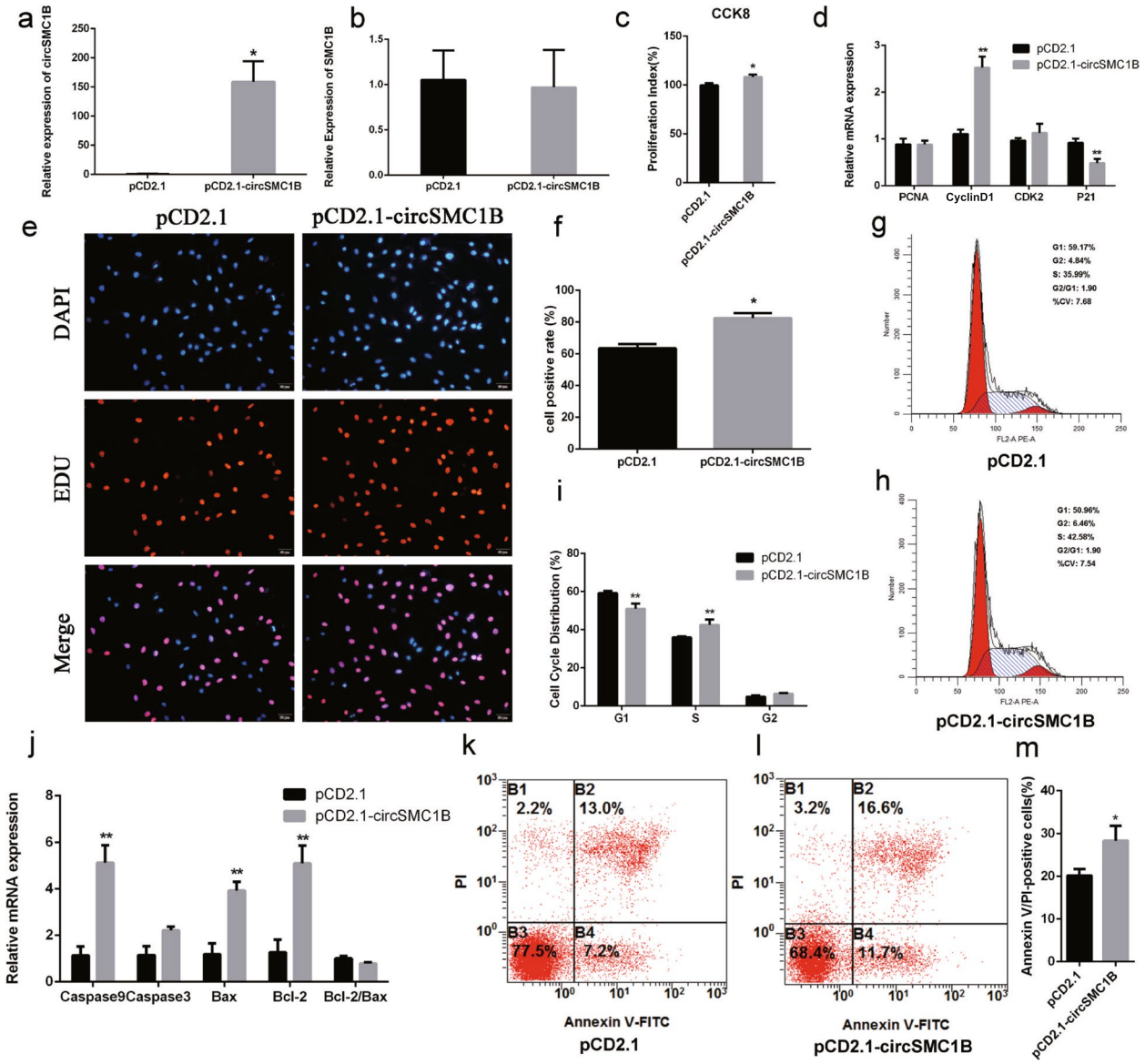

Fig. 3 Effect of circSMC1B on bovine mGSCs proliferation and apoptosis. **a**, **b** Visualization of pCD2.1-circSMC1B efficiency by RT-qPCR. **c** OD values were evaluated at 450 nm after CCK-8 addition. **d** *PCNA, CDK2, CyclinD1*, and p21 expression were detected by RT-qPCR. **e**-**f** The presence of EdU-positive cells was detected by the EdU kit in the proliferative phase. **g**-**i** Detection of cell cycle using flow cytometry and statistical analysis of cell numbers at various stages of the cell cycle, G1 phase, S phase. **j** After the addition of circSMC1B, detection of mRNA expression levels of cell apoptosis-related genes. **k**-**l** After circSMC1B activation, apoptotic mGSCs were detected by Annexin V-FITC/PI staining and flow cytometry. **m** Quantification of FITC-Annexin V/PI-positive cells presented in (**k**-**l**)

HMGA1 and *NR6A1* are targets of let-7i

To further clarify the potential molecular mechanism of let-7i in bovine mGSC proliferation and apoptosis, *HMGA1* and *NR6A1* were predicted as two potential target genes of let-7i using TargetScan7.2. *HMGA1* and *NR6A1* contain potential binding sites (13 and 7 bases, respectively) for let-7i in their 3' UTRs (Fig. 6a, c). Next, *HMGA1*-3'UTR and *NR6A1*-3'UTR (wild type-WT and mutant-MUT) luciferase reporter vectors were constructed, containing let-7i binding sites. Luciferase assay revealed that let-7i significantly decreased Rluc activity when co-transfected with let-7i mimic and psi-check2-*HMGA1/NR6 A1*-3' UTR-WT (Fig. 6b, e). Consistently, the expression of *HMGA1* and *NR6A1* was significantly inhibited at mRNA levels by overexpressing let-7i (Fig. 6c, f).

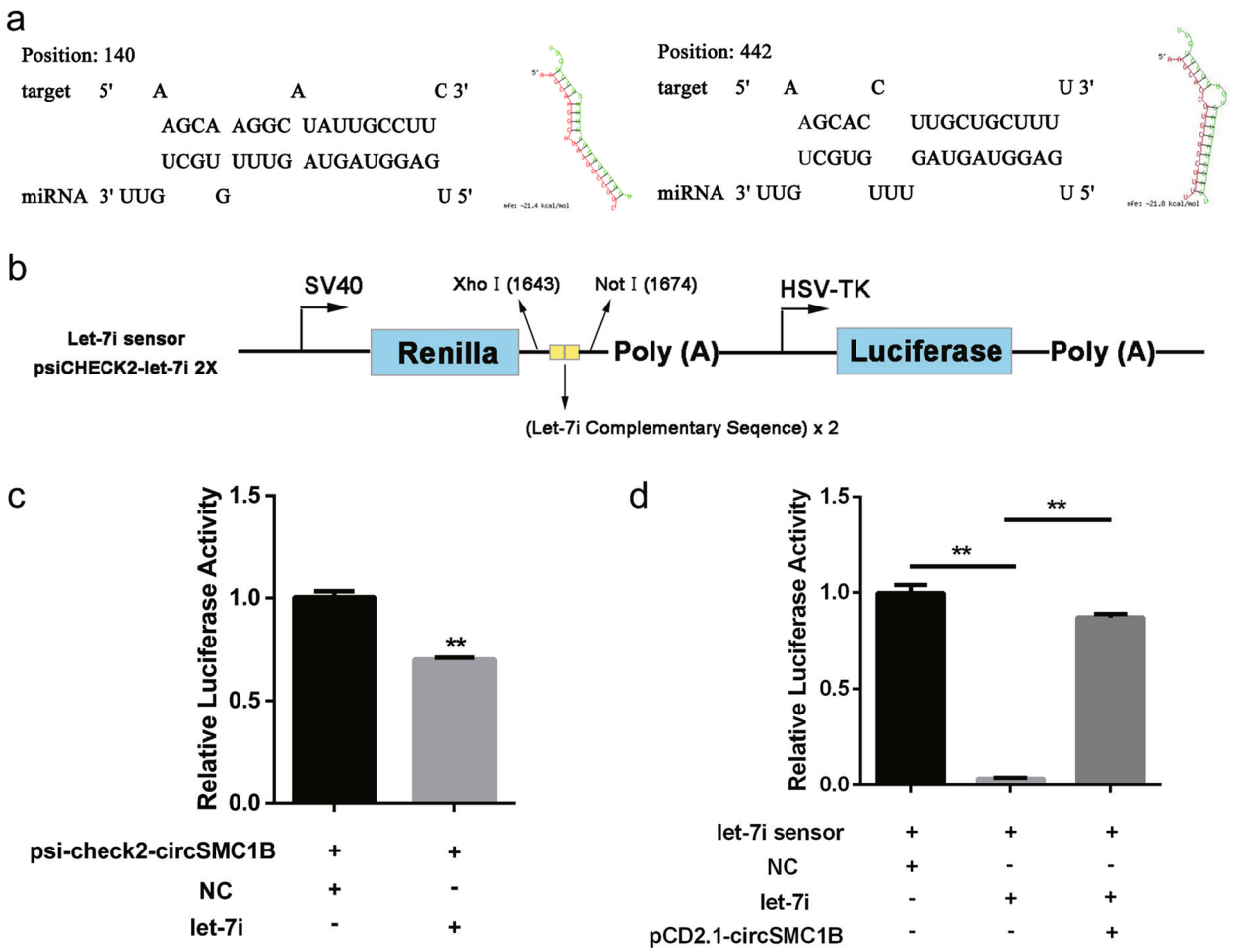

Fig. 4 CircSMC1B acts as a ceRNA for let-7i. **a** RNAhybrid predicted let-7i binding sites at two distinct positions in circSMC1B. **b** Schematic diagram of the let-7i sensor structure. **c** let-7i was co-transfected with psi-check2-circSMC1B into HEK293 T cells. **d** let- 7i sensors were co-transfected with let-7i mimic or circSMC1B into HEK293 T cells

CircSMC1B acts as a ceRNA for let-7i regulate bovine mGSCs proliferation and apoptosis

It has been demonstrated that let-7i can competitively bind to circSMC1B, and that circSMC1B promotes while let-7i inhibits mGSCs proliferation and apoptosis. Next, pCD2.1-circSMC1B and/or let-7i mimics were transfected into bovine mGSCs to see if the regulatory impact of circSMC1B on cell proliferation and apoptosis was dependent on the interaction with let-7i. At the mRNA level, we found that circSMC1B significantly increased *CyclinD1* expression; however, let-7i overexpression reversed these effects (Fig. 7a). With circSMC1B overexpression, the EdU-positive cells and mGSCs viability were significantly higher than the control group, and it trended significantly lower following co-transfection with let-7i (Fig. 7b-d). In a cell cycle experiment, it was observed that CircSMC1B exhibited an inhibitory effect on cell proliferation. Notably, the overexpression of let-7i mitigated these inhibitory effects exerted by CircSMC1B (Fig. 7e).

We wondered if circSMC1B could affect mGSCs apoptosis regulated by let-7i since let-7i can exert a protective effect against mGSCs apoptosis. The Hoechst 33,342 and PI dual staining assay showed that circSMC1B induced apoptosis in mGSCs and eliminated the anti-apoptotic effect generated by co-transfection with let-7i (Fig. 8b, c). Notably, let-7i also eliminated the increased expression of *Bax*, *Caspase3*, and *Caspase9* from the effect of overexpressed circSMC1B (Fig. 8a). Bovine mGSCs were treated with pCD2.1-circSMC1B and/or the let-7i mimic to demonstrate that circSMC1B functions as a ceRNA to alleviate the miRNA inhibitory impact on *HMGA1* and *NR6A1*(Fig. 8d). Results from RT-qPCR revealed that circSMC1B significantly upregulated the expression of *HMGA1* and *NR6A1* and that let- 7i overexpression reversed this effect.

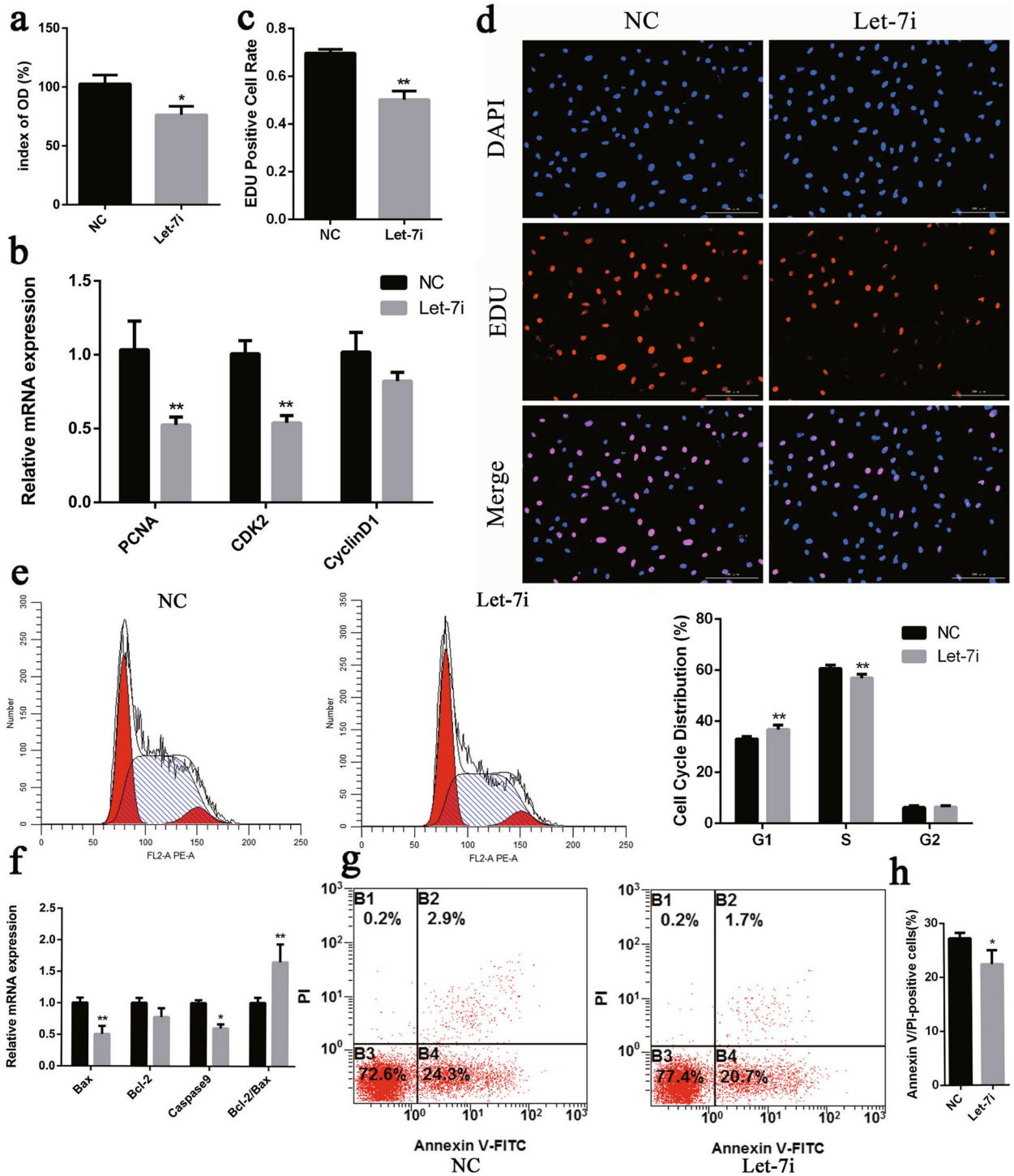

Fig. 5 Effect of let-7i on bovine mGSCs proliferation and apoptosis. **a** OD values measured at 450 nm after CCK-8 addition. **b** PCNA, CDK2, and CyclinD1 expression levels detected by RT-qPCR. **c**-**d** The occurrence of EdU-positive cells was detected by the EdU kit in the proliferative phase. **e** Detection of the cell cycle using flow cytometry and statistical analysis of cell numbers at various stages of the cell cycle, G1 phase, S phase and G2 phase. **f** After a gain of let-7i, detection of mRNA expression levels of cell apoptosis-related genes. **g** After the induction of let-7i, apoptotic mGSCs were detected by Annexin V-FITC/PI staining and flow cytometry. **h** Quantification of FITC-Annexin V/PI-positive cells presented in (**g**)

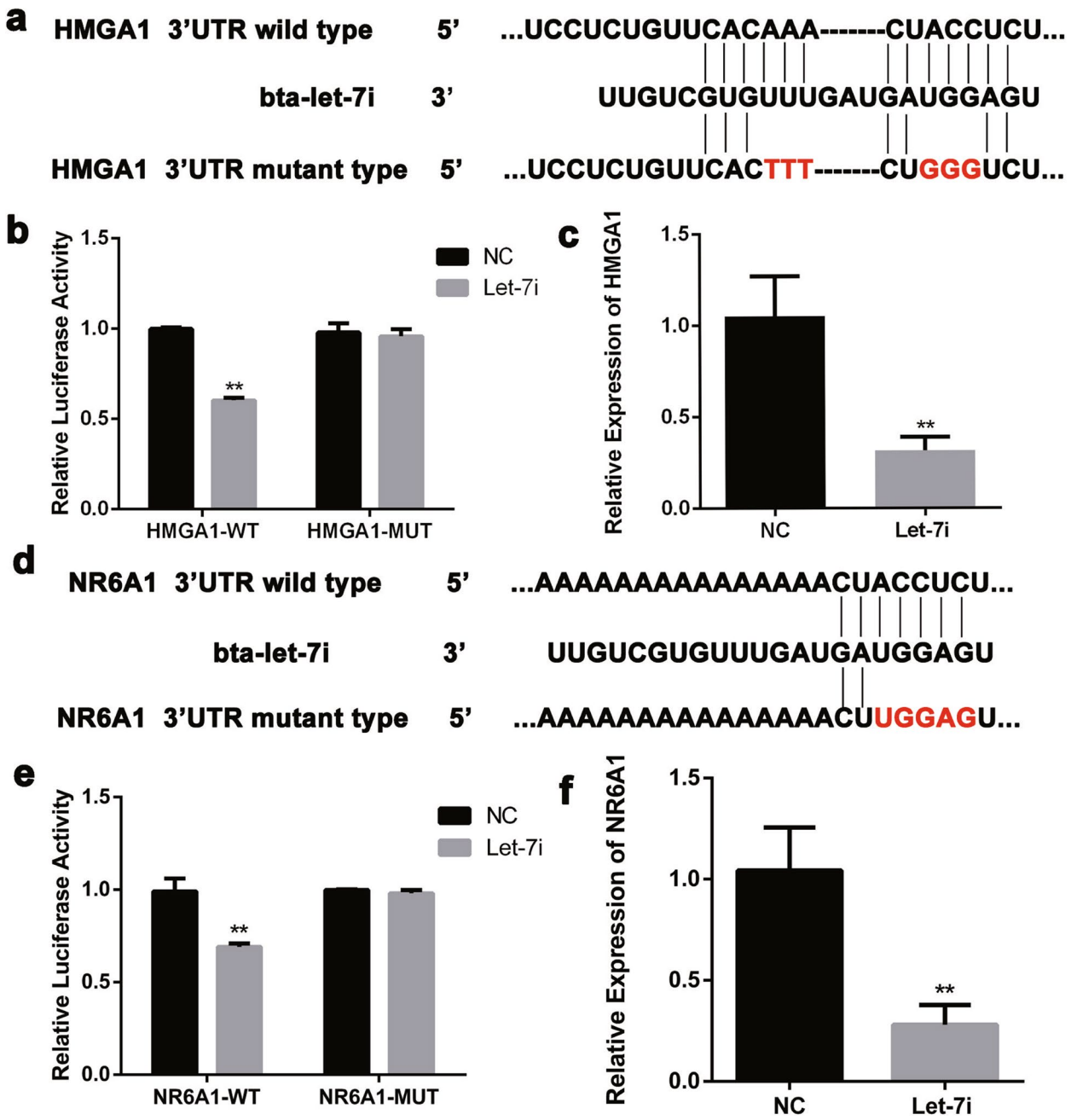

Fig. 6 Verification of the let-7i target gene. **a**, **d** The predicted binding sites of let-7i in the 3'UTR of *HMGA1* or *NR6 A1*. **b**, **e** The wild-type or mutant of *HMGA1* or *NR6A1* dual-luciferase vector and let-7i mimic was co-transfected into HEK293 T cells; (**c**, **f**) Overexpressing let-7i mimics suppressed the expression of the target gene of *HMGA1* and *NR6A1*

Taken together, our findings demonstrate that circ-SMC1B promotes bovine mGSCs proliferation and apoptosis by sequestering let-7i.

Discussion

The primary function of the testis is to produce spermatozoa and facilitate hormone synthesis [37]. Testicular

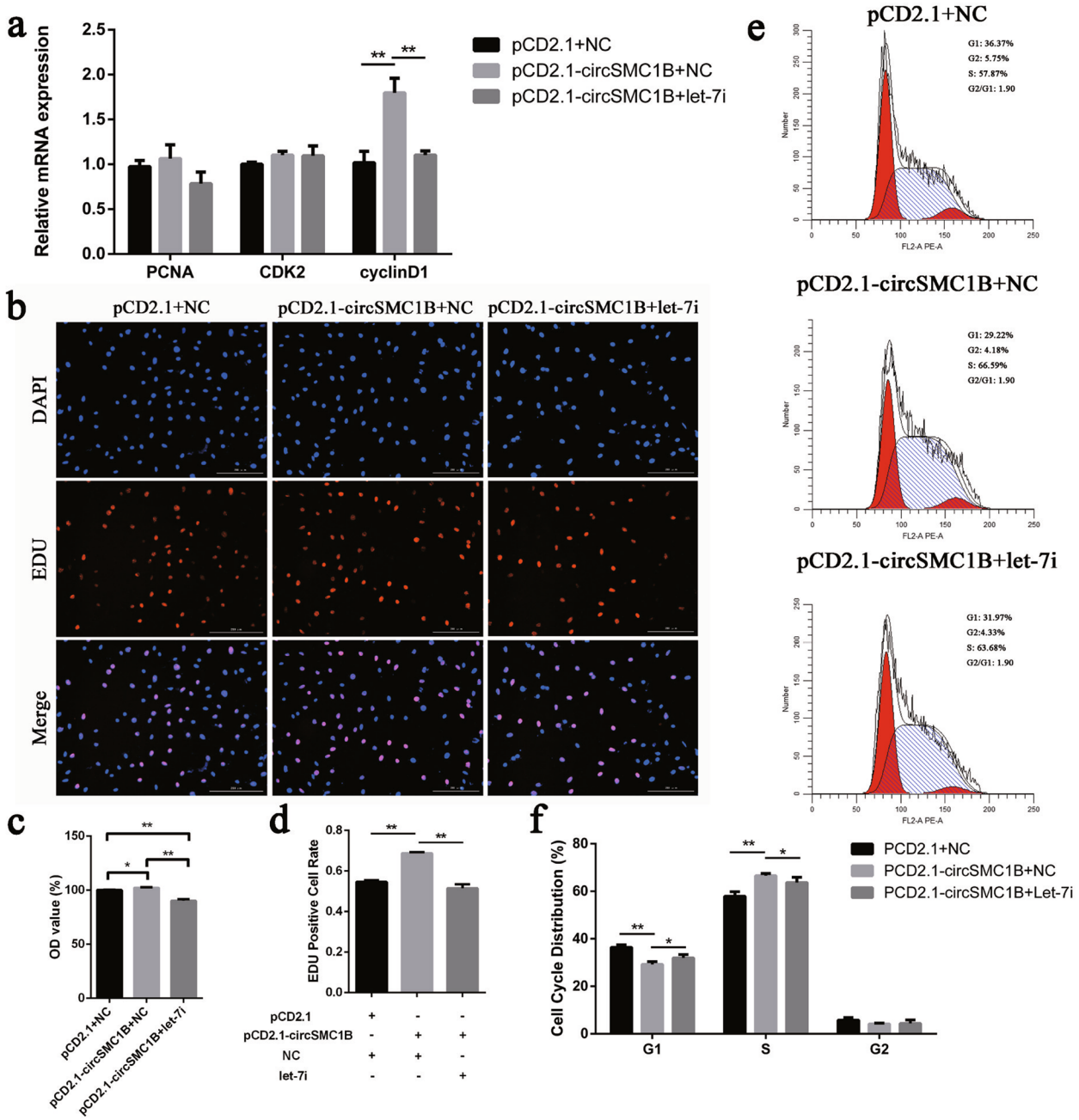

Fig. 7 CircSMC1B binding let-7i to promote mGSCs proliferation. **a** *PCNA*, *CDK2*, and *CyclinD1* expression was detected by RT-qPCR. **b**, **d** The presence of EdU-positive cells was detected by the EdU kit in the proliferative phase. **c** OD values were evaluated at 450 nm after CCK-8 addition. **e**, **f** Detection of the cell cycle using flow cytometry and statistical analysis of cell numbers at various stages of the cell cycle, G1 phase, S phase, and G2 phase

development and spermatogenesis are heavily influenced by the proliferation, differentiation, and apoptosis of various germ cells [38]. These complex processes require precise regulation of numerous genes and networks, which can cooperate or antagonize each other at both transcriptional and post-transcriptional levels [39, 40]. The reproductive efficiency of breeding bulls is crucial for achieving desired genetic traits, which are achieved through the selective use of germ cells from superior sires [41]. Enhancing the reproductive efficiency of bulls through genetic selection is of utmost importance, as a single bull can impregnate multiple cattle via artificial

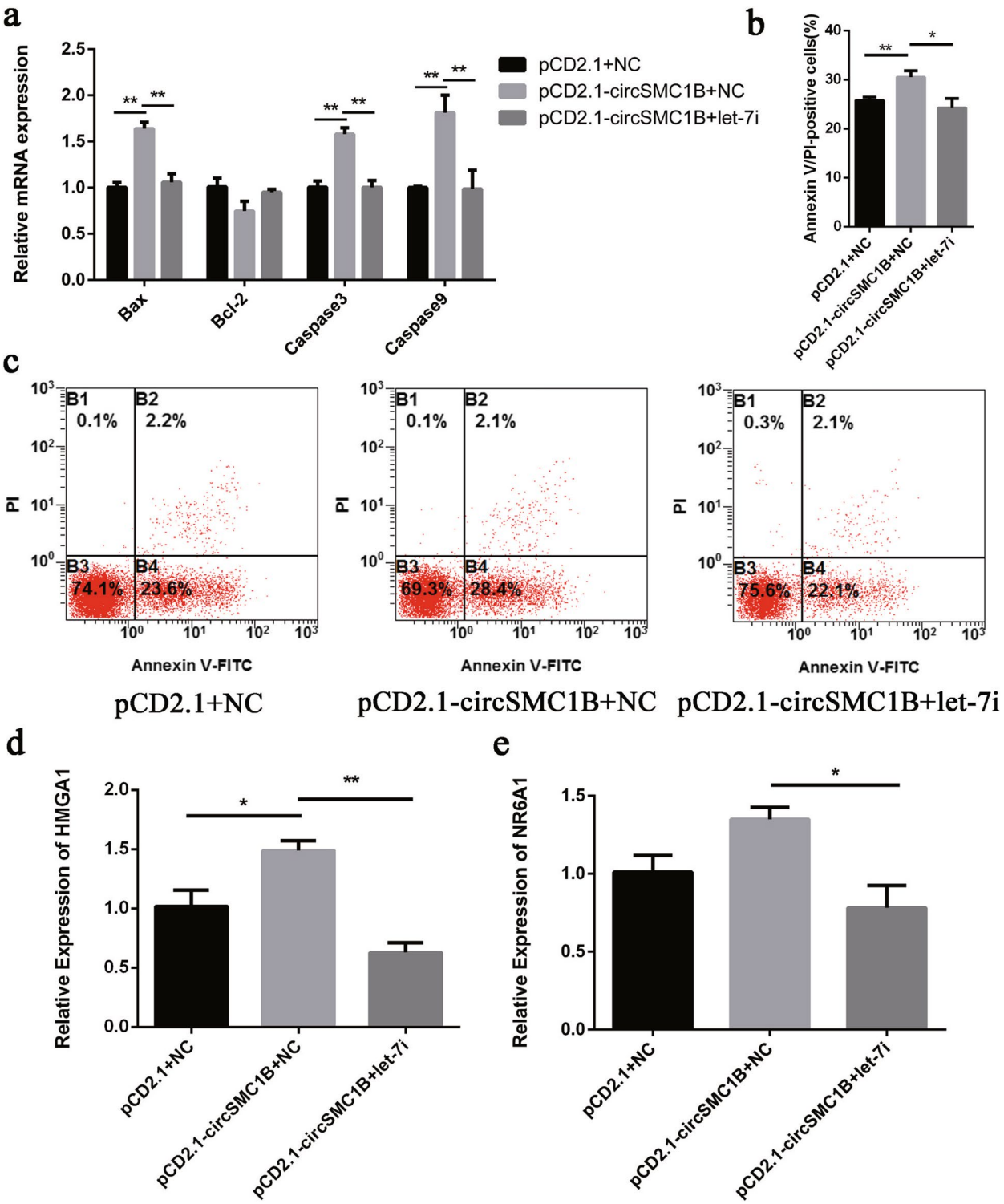

Fig. 8 CircSMC1B binding to let-7i promotes mGSCs apoptosis and let-7i target genes expression. **a** Detection of mRNA expression levels of cell apoptosis-related genes. **b** Quantification of FITC-Annexin V/PI-positive cells presented in (**c**). **c** Apoptotic mGSCs were detected by Annexin V-FITC/PI staining and flow cytometry. **d**, **e** circSMC1B significantly promotes the expression of *HMGA1* and *NR6A1*, whereas let-7i overexpression abrogates this effect

insemination [42]. Understanding the regulatory mechanisms underlying testicular development and spermatogenesis is crucial for advancing cattle breeding. Previous studies have linked circRNAs to the male reproductive system and infertility [43]. However, these studies have limitations, including a small number of animal samples and a wide age range of the sampled animals [18, 21]. Thus, this study aimed to investigate the influence of circRNAs on the developmental processes of bovine testes, focusing on the period from birth to sexual maturity.

In this study, a total of 987 circRNAs were differentially expressed in the bovine testis during the neonatal and mature stages (P-adjust <0.05). Compared to the neonatal stage, 710 circRNAs were up-regulated and 277 circRNAs were down-regulated in the mature stage. This finding aligns with previous reports that observed a higher number of upregulated circRNAs in older bulls. Interestingly, the number of circRNAs detected in Angus bull testes from birth to sexual maturity was lower than that in Qinchuan bull testes from birth to body maturity. This study specifically focused on identifying circRNAs associated with bovine sexual maturity. Given the high expression and stability of circRNAs in the testes, it is plausible that these RNAs play a crucial role in spermatogenesis and the pathogenesis of infertility-related diseases. Furthermore, the host genes of these differentially expressed circRNAs were enriched in male reproductive pathways, suggesting that circRNAs significantly contribute to cattle testis development and spermatogenesis.

A study by Zhang et al. reported that some of these differentially expressed circRNAs are involved in signaling pathways regulating pluripotency in stem cells, such as fibroblast growth factor receptor 1 (*FGFR1*), AKT serine/threonine kinase 3 (*AKT3*), SMAD family member 4 (*SMAD4*), Frizzled Class Receptor 3 (*FZD3*), and Activin A receptor type 1 (*ACVR1*) that regulate pluripotency in stem cells [20]. Additionally, circRNAs were found to be highly expressed in spermatogenic cells. A previous study identified 15,101 circRNAs in mouse spermatogenic cells, with 7,220 expressed in round spermatids and the rest in SSCs (5,573), spermatogonia type A (5,596), preleptotene (6,686), and pachytene spermatocytes (4,677) [44]. However, further experiments are necessary to determine the true impact of these circRNAs on testicular development and sperm production. Investigating whether circRNAs affect cell proliferation, differentiation, and apoptosis in the testis will help elucidate the molecular mechanisms underlying circRNA function.

Among ncRNAs, circRNA is considered more stable than other RNA molecules due to the absence of 5'-3'polyadenylated tails. CircRNAs have been identified as biomarkers in various diseases, including colon cancer [45], and Alzheimer's disease [46]. While research on testicular tissue growth and development has primarily focused on protein-coding genes, miRNAs, and lncRNAs in recent years, there has been limited research on circRNAs in testis development. This study aimed to address this research gap by verifying candidate circRNAs in Qinchuan cattle using RT-qPCR and comparing them with the existing circRNA database [18]. Specifically, circSMC1B (bta_circ_0021665), a highly expressed circRNA in testicular tissues and mGSCs, was selected for further exploration of its functions. Systematic assays revealed that circSMC1B promoted bovine mGSCs proliferation and apoptosis. Interestingly, the trends of cell proliferation and apoptosis were consistent, contrary to the typical pattern where these processes oppose each other [47]. To explain this phenomenon, we identified two potential reasons based on existing literature. Firstly, germ cells undergo apoptosis during spermatogenesis [48, 49]. Accelerated stem cell proliferation can expedite the entire spermatogenesis process, leading to an increase in apoptotic cells. Second, it has been reported that the ratio of degenerated germ cells in cattle during spermatogenesis is higher than in other species [50]. In humans, there is a 30% to 40% reduction in potential sperm production towards the end of meiosis [51]. In bulls, significant degenerated germ cells are observed during spermatocytogenesis (mitosis), with approximately 30% of germ cells lost between A and intermediate spermatogonia, and another 30% lost between B1 and B2 spermatogonia [52, 53]. These observations provide a reasonable explanation for the consistent trends of proliferation and apoptosis seen in our experimental results, suggesting that the reduced cellular degradation during spermatocytogenesis may contribute to the increased productivity of bull spermatozoa.

Several studies have highlighted the regulatory role of circRNAs in male infertility through their interactions with miRNAs. For instance, hsa_circ_0023313, upregulated in NOA patients, may regulate spermatogenesis through the hsa_circRNA_0023313/miR-372-3p/RAB-24 and/or hsa_circRNA_0023313/miR-373-3p/USP-24 pathways [54]. Another circRNA, hsa_circRNA_402130, may play a pathological role in NOA pathogenesis by acting as a miRNA"sponge"for the let-7 family [55]. Additionally, loss of circSRY has been shown to impair mouse spermatogenesis by reducing γH2 AX levels through sponging miR-138-5p in germ cells [56]. These studies strongly indicate the significance of circRNAs acting as miRNA sponges. Our study further contributes to this understanding by demonstrating that let-7i overexpression inhibits mGSCs proliferation and apoptosis, suggesting that it acts as an inhibitory factor. Xu et al. identified 27 differentially expressed miRNAs in cattle, yak, and cattle-yak spermatogenic cells, with bta-let-7 family,

bta-miR-125, and bta-miR-23a downregulated, potentially impairing spermatogonia differentiation induced by RA [57]. Shen et al. found that down-regulating let-7 can lead to early spermatogonia differentiation into primary spermatocytes, whereas inhibiting let-7 expression enhances differentiation [58]. The let-7 family is known to regulate stemness through its interaction with LIN28A and has been found to impact the spermatogenesis process in mice by reducing the germ cell pool [59]. This suggests that down-regulation of let-7 inhibits spermatogonia differentiation. In our study, circSMC1B was found to sponge let-7i, reducing its inhibition of *HMGA1* and *NR6A1*, thereby promoting bovine mGSCs proliferation and apoptosis in vitro. Overexpression of let-7i reversed this effect. Thus, our findings demonstrate that circSMC1B indirectly regulates the transcription of *HMGA1* and *NR6A1*, influencing the proliferation and apoptosis of mGSCs.

The significance of *HMGA1* in normal sperm production in mice was revealed through the construction of *HMGA1* chimeric mice by Liu et al. These mice exhibited reduced seminiferous tubule diameter, vacuolated supporting cells, and a lack of sperm, highlighting the necessity of the *HMGA1* allele for normal spermatogenesis [60]. Moreover, *HMGA1* has been identified as a marker gene for spermatogonia in human single-cell sequencing [61]. Another gene of interest, *NR6A1* (also known as *GCNF*) predominantly expressed in round spermatozoa after meiosis at both mRNA and protein levels in the mouse testes [62–64]. In human testes, *NR6A1* is expressed in pachytene sperm cells [65]. *NR6A1* functions as a transcription factor to regulate the transcription of genes, including protamine genes, after meiosis, potentially influencing male fertility [66–68]. Collectively, *HMGA1* and *NR6A1* play critical roles in spermatogenesis. However, further investigation is required to elucidate these mechanisms in bovine testes development. In summary, our findings demonstrate that circSMC1B functions as a ceRNA to regulate bovine mGSCs proliferation and apoptosis.

Conclusions

This study aimed to characterize circRNAs in Angus cattle testis at neonatal and sexual mature stages. A total of 28,065 candidate circRNAs were identified, out of which 987 circRNAs exhibited differential expression (*P*-adjust < 0.05). Among these, 710 circRNAs were up-regulated and 277 circRNAs were down-regulated in the sexually mature stage compared to the neonatal stage. Notably, circSMC1B exhibited high expression levels in both the sexually mature stage and bovine mGSCs. Through multiple experiments, we demonstrated that circSMC1B acts as a regulator of target genes *HMGA1* and *NR6A1* by binding let-7i. Furthermore, circSMC1B was found to promote bovine mGSCs proliferation and apoptosis, implicating its involvement in enhancing bovine spermatogenesis as a ceRNA. These findings provide valuable insights into the transcriptional regulatory mechanisms underlying bull testis development and spermatogenesis.

Abbreviations

circRNAs	Circular RNAs
ncRNA	Non-coding RNA
cDNA	Complementary DNA
DE	Differentially expressed
OD	Optical density
mGSCs	Male germline stem cells
RT-qPCR	Quantitative real-time PCR

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11556-3.

Additional file 1: Table S1. Primer list for vector construction. Table S2. Primer list for RT-qPCR. Table S3. Information on circRNAs. Table S4. Information on differentially expressed circRNAs. Table S5. Read count information on differentially expressed circRNAs in all samples. Table S6. Enriched GO terms for differentially expressed circRNAs. Table S7. Enriched the KEGG pathways for differentially expressed circRNAs (Top 20).

Additional file 2.

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Authors' contributions

YG: Conceptualization, Methodology, Investigation, Writing-Original draft. CL: Visualization, Writing-Review & Editing. HJ: Writing-Review & Editing. GY, ZW: Data curation. CZL: Conceptualization, Methodology, Supervision. RD: Conceptualization, Funding acquisition.

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Data availability

The raw sequence data reported in this paper have been deposited in the Sequence Read Archive database (SRP148084), https://www.ncbi.nlm.nih.gov/sra. All data generated and analyzed in this study are available in the article and the Supplementary materials.

Declarations

Ethics approval and consent to participate

All animal work was approved by the ethics committee of Northwest A&F University (Yangling, Shaanxi, China) (Permit No. NWAFAC1019). Furthermore, the care and use of experimental animals were fully compliant with local animal welfare laws, guidelines, and policies.

Consent for publication

Not applicable.

Competing interests
The authors declare no competing interests.

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