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LncRNA Slfn5os Regulates the Survival and Testosterone Production in Tm3 Leydig Cell Lines

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Abstract

Long non-coding RNAs (lncRNAs) have been reported to regulate the spermatogenesis. In this study, we aim to characterize the expression pattern and roles of lncRNA schlafen 5, opposite strand (Slfn5os) in the testis of adult mouse. The expression of lncRNA Slfn5os and Slfn5 in different tissues was examined by Reverse-Transcription Polymerase Chain Reaction (RT-PCR). The localization of lncRNA Slfn5os and Slfn5 was determined by Fluorescence In situ Hybridization (FISH). TM3 Leydig cell line was used as a cellular model to study the function of Slfn5os. The survival of TM3 cells upon Slfn5os knockdown or overexpression was assessed by Cell Count Kit-8 (CCK-8) viability assay and flow cytometry. The secretion of testosterone by TM3 cells was detected by Enzyme-Linked Immuno Sorbent Assay (ELISA). We found that Schlafen family member 5 (Slfn5) was widely expressed in all the tissues examined, while lncRNA Slfn5os was exclusively expressed in the testis. FISH staining revealed a Leydig cell-specific expression of Slfn5os. The expression level of Slfn5os negatively regulates the mRNA level of Slfn5. In addition, forced expression of Slfn5os impaired the survival and testosterone production in TM3 cells, while Slfn5os silencing showed the opposite effects. The reduced testosterone production upon Slfn5os overexpression was associated with the down-regulation of steroidogenic genes. We conclude that Slfn5os is a testis-enriched lncRNA in mouse testis, which regulates the survival and testosterone production in Leydig cells.

Keywords: LncRNA; Slfn5os; Testis; Testosterone; Leydig cells

Introduction

Spermatogenesis is a male reproductive process in testis responsible for the production of spermatozoa from spermatogonial stem cells, which comprises of mitosis, meiosis and spermiogenesis [1]. The meiosis of spermatogonia generates haploid spermatids, and the spermatids eventually differentiate into mature spermatozoa [2]. Sertoli cells are a group of nursing cells surrounding the spermatogonia which release neurotrophic factors, retinoic acid and growth factors to support spermatogenesis [3]. Except for the spermatogenesis, the testis is also responsible for androgen production. Leydig cells are the main source of androgens such as testosterone through the steroidogenic pathway to stimulate spermatogenesis by regulating Sertoli cells [4, 5]. The malfunction of Leydig cells and the insufficient level of testosterone could underline the spermatogenesis in the testis [6, 7]. For example, neuregulin-1 depletion in mice impairs Leydig cell proliferation and testosterone production in the testes, leading to defective spermatogenesis and a reduction of testis weight [7].

Androgen production is regulated by a hierarchical axis of hypothalamus-pituitary-gonad [8]. Gonadotropin-Releasing Hormone (GnRH) secreted by the hypothalamus induces the production of Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH) from the pituitary, which in turns stimulates the production of testosterone in Leydig cells [9, 10]. The steroidogenesis (the biosynthesis of steroids such as steroid hormone) in Leydig cells can be controlled by autocrine and paracrine factors within the testis [10, 11]. Although the endocrine system responsible for steroidogenesis is well-established, the molecular mechanisms underlying the transcriptional control of spermatogenetic genes remain to be fully elucidated.

Non-coding RNAs (ncRNAs) have recently been highlighted as another layer of gene expression control in sex determination and sex organ development [12]. ncRNAs can be roughly classified into two categories based on the length: small ncRNAs (20-50 nucleotides) and long ncRNAs (lncRNAs) (> 200 nucleotides) [13]. Regarding small ncRNAs in the testis, microRNAs and Piwi-interacting RNAs have been well characterized in the post-transcriptional regulation of mRNAs during spermatogenesis [14, 15]. Recently, the roles of different lncRNAs as gene expression regulators in spermatogenesis have attracted research attention, although the determination of the expression profiles and roles of lncRNAs in testis can be challenging [16, 17]. For example, 1700108J01Rik and 1700101O22Rik have been identified as two testis-associated lncRNAs [18], and lncRNA Start has been reported as a regulator of steroidogenesis in Leydig cells [19]. However, the regulatory mechanisms and the molecular targets of the majority of lncRNAs in testis remain unclear.

Slfn5os (schlafen 5, opposite strand, 1700003F17Rik) is a novel lncRNA identified in mouse testis, and it has been found to be regulated by an endocrine disruptor, Di-2-Ethyl Hexyl Phthalate (DEHP) [20]. It is transcribed from the opposite strand of Slfn5 (Schlafen family member 5) gene. However, its tissue-specific expression and functional role in testis is largely unknown. Elucidating its expression pattern and role in testis can provide insights into the novel mechanism of spermatogenesis regulation. In this study, we aim to characterize the tissue-specific expression pattern of Slfn5os, clarify its localization in testis, investigate its impact on the expression of Slfn5, examine its role in the survival and testosterone production of Leydig cells, as well as the role in regulating key genes/enzymes in steroidogenesis.

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Material and Methods

Animals and cell culture

Eight-week-old male C57BL/6 mice were used in this study. All animal experiment procedures were approved by the Animal Experimentation Ethics Committee of Jilin University. Mice were housed in a germ-free animal house at a 12-hour light/12-hour dark cycle with free access to food and water. All mice were sacrificed under ketamine anesthesia to minimize the discomfort.

The TM3 Leydig cell line and immortalized murine spermatocyte cell line GC-2spd(ts) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The TM3 Leydig cell line were cultured in a 1:1 (v:v) mixture of DMEM:F12 media supplemented with 1% penicillin/streptomycin (Invitrogen, CA, USA), 2.5% fetal bovine serum and 5% horse serum (Sigma) at 37°C with 5% CO_2. GC-2spd cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma) and 1% penicillin/streptomycin (Invitrogen, CA, USA).

Cell transfection

Si-Slfn5os (siRNA targeting Slfn5os), negative controls (si-NC), pcDNA. 3.1 empty vector and pcDNA3.1-Slfn5os overexpression plasmid were purchased from MDL Bio (Beijing, China). For transfection, TM3 cells were seeded in a 6-well plate at 1×10^5 cells per well for 24 h before transfection. Prior to transfection, the cell culture medium was replaced with serum-reduced medium (Opti-MEM; Invitrogen, USA). Lipofectamine 2000 (Invitrogen, USA) was used to transfect TM3 cell according to the protocols supplied by manufacturers. In 6-well plates, 60% confluent cells were transfected with 50 nM of siRNA or 6 μg of plasmid. 8 h after transfection, the medium was replaced with serum-containing medium and the transfected cells were subjected to subsequent analysis 48 h post-transfection.

Fluorescence *In situ* Hybridization (FISH)

Adult mouse testes were fixed for 3 h at 4°C with 4% paraformaldehyde in Phosphate Buffered Saline (PBS), and sliced into 5 μm thick sections. Hybridization was performed according to a previously reported protocol [21]. After hybridization and washing, the sections were incubated with anti-DIG horseradish peroxidase antibody (1:500 dilution; Roche) overnight at room temperature. The reaction with tyramide-Cy3 (1:50 dilution in 1X Plus Amplification Diluent [PerkinElmer], followed by 1:100 dilution with distilled water) was performed at room temperature for 1h. All sections were counterstained with a DAPI-containing medium and mounted with a Fluoro-KEEPER Antifade Reagent (Nacalai Tesque, Kyoto, Japan). The samples were observed under an LSM 5 LIVE confocal microscope (Carl Zeiss, Oberkochen, Germany). The sequences of the probe were as follows:

Slfn5os:5'-TTATAGAAGAGACGAGGAAGGACTAGTG CC-3'-(30 base pairs)

Slfn5: 5'-AGGCATCGATAGTAGATTTCAAGTT-3'-(25 base pairs)

Quantitative RT-PCR and RT-PCR (reverse-transcription PCR)

Total RNA was extracted from the tissue and cells using Trizol reagent (Invitrogen, Waltham, MA), according to the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed into cDNA with random primers (Invitrogen) and Avian Myeloblastosis Virus Reverse Transcriptase (Promega Corp., Madison, WI). The resulted cDNA was analyzed in a 7500 Real Time PCR System (Applied Biosystemss, Carlsbad, CA, USA) using SYBR premix EX TAQ II kit (Takara, Dalian, China). The PCR cycling condition used: 95°C 2 mins, 40 cycles of 95°C 30 sec, 60°C 30 sec and 72°C 60 sec. $2^{-\Delta\Delta Ct}$ method was used to analyze the relative expression level and actin was used as the internal reference gene.

For RT-PCR, the cDNA was amplified with Slfn5os and Slfn5 primers. PCR consist of 42 cycles of denaturation at 94°C for 45 sec, annealing at 57°C for 1 min and extension at 72°C for 90 sec. The amplified products were separated by electrophoresis in 1% agarose gels and stained by ethidium bromide. The primer sequences of Real-time quantitative PCR and RT-PCR primers were listed in Table 1, which were synthesized by Operon Technologies (Alameda, CA, USA). PCR primers used for Sfln5os amplification target the unique region of Sfln5os, which does not overlap with nearby genes such as Slfn5 and Unc45b.

Cell proliferation assays

Cell Counting Kit-8 (CCK-8; Fluorescence Biotechnology) was used for cell proliferation assay. Cells were seeded in 96-well plates at the density of 5000 cells per well. After 48 h, 10 μL of CCK-8 solution was added into each well and the cells was incubated in a humidified incubator for 1 h at 37°C. Then DMSO was added into each well, followed by agitation on a swing table at a low speed for 10 min to fully dissolve the crystals. The light absorption value (OD value) in each condition was captured at 490 nm wavelength in a microplate reader (Bio-Rad, Richmond, CA, USA).

Apoptosis detection by flow cytometry

Apoptosis evaluation of TM3 cell was performed by an Annexin V and PI staining Kit (4A Biotech Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions. Cells were adjusted to 1 × 106 cells/mL in staining buffer. 5 μL Annexin V-FITC and 5 μL PI solution were added to the cell suspension and incubated for 30 min in the dark. Stained cells were centrifuged and washed twice with

Table 1: Primer sequences.

Gene	Primer Sequence
Slfn5 (RT-PCR)	F: 5' TGATGGTGGGTGAAGAA 3' R: 5' GCAACATCCGTGAAATAG 3'
GAPDH (RT-PCR)	F: 5' TGGCCTTCCGTGTTCCTAC 3' R: 5' GAGTTGCTGTTGAAGTCGCA 3'
Slfn5os (RT-PCR)	F: 5' GTGCTGCACGGACAACT 3' R: 5'TGCCTCCTCACTGATTCTT 3'
Slfn5 (qPCR)	F: 5'GAGCCCAGTTTACTAGTGCAG3' R: 5'TTTCCATGCCTGAAAATCGG3'
Star (qPCR)	F: 5' AGCTCCTATAGACATATGCGGAA3' R: 5' AGAACCAAGCAGAGAGCTCC 3'
Cyp11a (qPCR)	F: 5' AGCTGCCCTTCAAGAACATCCA 3' R: 5' CCGCAGCATCTCCTGTACCTT 3'
Cypl7al (qPCR)	F: 5' TACCGTTTCTCCCCAGACGTG3' R: 5' CCAGCTGATAGTGACCGACA3'
Lhr (qPCR)	F: 5' AAAGCACAGTTAGAGAAGCGAAT 3' R: 5' GCCTCAGCTTTTGGTCCAG3'
β-Actin (qPCR)	F: 5' TCCTCCTGAGCGCAAGTACTCC 3' R: 5' CATACTCCTGCTTGCTGATCCAC3'
Hsd3b1 (qPCR)	F:5'CTCTGGACAAAGTATTCCGAC3' R:5'TGGGCATCCAGAATGTCTC3'
Hsdl7b3 (qPCR)	F:5'CATGAGGTTCTCGCAGCAC3' R:5'AATCACTGCCCATTGTCCC3'

staining buffer and re-suspended in 400 μL staining buffer. Stained cells were analyzed using BD FACS CantoTM II Flow Cytometer (BD Biosciences, CA, USA).

Testosterone measurement by ELISA (Enzyme-Linked Immuno Sorbent Assay)

Testosterone concentrations were determined from supernatant of cell culture a testosterone ELISA kit (Elabscience Biotechnology Co.,Ltd, Wuhang, China) according to the manufacturer's instructions. 25 mIU/ml hCG (human Chorionic Gonadotropin) was added to the cell medium to induce testosterone biosynthesis. Briefly, 100 μL supernatant was added to the capture-antibody-coated plate. After a wash step to remove unbound material, biotin-labeled detection antibody was added for 1 h incubation, which was followed by staining with streptavidin–HRP (Horse Radish Peroxidase) for 1 h at room temperature. Chemiluminescent detection reagents were added for signal development at room temperature for 15 min and the optical density of samples and standards was measured at 450 nm in a microplate reader (Bio-Rad, Richmond, CA, USA). The serum containing fresh medium was used as the blank sample, from which all the other readings were subtracted. The concentration of Testosterone in each sample was determined based on the linear regression of the standards.

Western blotting

Total protein was extracted from cells using RIPA lysis buffer containing protease inhibitor cocktail (Thermo Fisher Scientific, MA, USA) 4°C for 15 min. The cell lysates were centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant containing total protein lysate was quantified by a BCA Protein assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Protein samples (30μg) were separated by 10% SDS–PAGE (Sodium Dodecyl-Sulfate Poly Acrylamide Gel Electrophoresis) and then transferred to PVDF (Poly Vinylidene Di Fluoride) membrane (Millipore, CA, USA). The membrane was blocked with 10% skimmed milk for 1 h at room temperature and incubated with the primary antibodies: rabbit anti-mouse antibodies to LHCGR (Luteinizing Hormone/Chorionic Gonadotropin Receptor) (1:1000, cat. 19968-1-AP, Proteintech, USA), STAR (Steroidogenic Acute Regulatory Protein) (12225-1-AP, diluted at 1:1500, Proteintech, USA), CYP11A1 (Cytochrome P450 Family 11 Subfamily A Member 1) (13363-1-AP, diluted at 1:2000, Proteintech, USA) and Actin (66009-1-Ig, diluted at 1:2000, Proteintech, USA) at 4°C overnight. The membrane was washed 3 times, and further incubated with HRP-linked secondary antibody (1:3000 dilutions, Cell signaling technologies #7074, MA, USA) at room temperature for 1 h. Then the membrane was washed 4 times with TBST buffer and the protein bands were visualized using an enhanced chemiluminescence kit (Santa Cruz, TX, USA) and photographed on a gel imager system (Bio-Rad, Hercules, CA, United States). The densitometry analysis was performed with Image J software (Bethesda, MD, USA).

Statistical analysis

All experiments were performed at least three times in this study, and all data are presented as the mean ± Standard Error of the Mean (SEM). All statistical analyses were performed with Graphpad 8.0 (GraphPad Software, San Diego, CA, USA). The statistical difference between two groups was compared using unpaired Student's t tests. Comparisons among multiple groups were analyzed using one-way Analysis of Variance (ANOVA) with Tukey's post hoc test for pair-wise comparison. Comparisons of data at multiple time points were examined using two-way ANOVA. A P-value < 0.05 was considered statistically significant.

Results

Testis-specific expression of Slfn5os in Leydig cells

We first sought to determine the tissue-specific expression pattern of Slfn5 and the antisense lncRNA Slfn5os. Total RNAs were prepared from different tissues of adult mice, including the heart, brain, lung, spleen, kidney, ovary, uterus and testis. RT-PCR was performed to examine the expression pattern of Slfn5 and antisense Slfn5os. The results showed that Slfn5 was widely expressed in all the tissues examined, while lncRNA Slfn5os was exclusively expressed in the testis (Figure 1A).To further determine the localization of Slfn5 and Slfn5os in testis, we performed Fluorescence *In situ* Hybridization (FISH) with a highly sensitive tyramide signal amplification system. Probes for Slfn5or Slfn5os were used for FISH in the sections of adult testis. As shown in Figure 1B, strong signals for Slfn5were detected in the Leydig cells. In contrast, weaker staining signals of Slfn5os were detected in the cytoplasm of Leydig cells (Figure 1B). Taken together, these results demonstrate that Slfn5os is uniquely expressed in the cytoplasm of Leydig cells.

The impact of Slfn5os on Slfn5 expression

In order to investigate whether Slfn5os could affect the expression of Slfn5 in testis, two mouse cell lines spermatocytes (GC-2) and

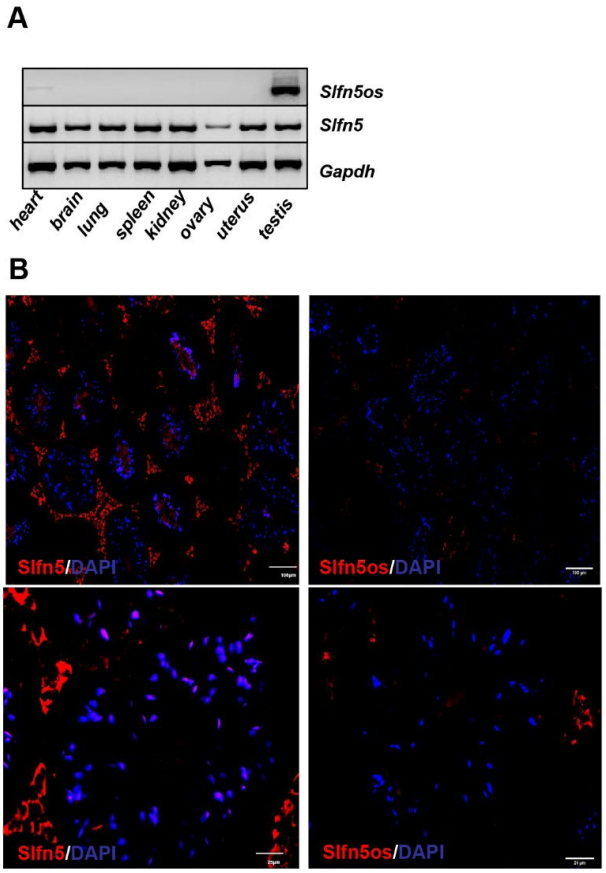

Figure 1: Expression pattern and localization of Slfn5 and lncRNA Slfn5os in adult mouse testis. (A) The expression levels of Slfn5 and antisense Slfn5os were examined by RT-PCR in heart, brain, lung, spleen, kidney, ovary, uterus, testis tissues of adult mice. (B) FISH examination of Slfn5 and Slfn5os in adult mouse testis sections. (Scale bar, 100 μm or 25 μm).

Leydig cells (TM3) were selected. Consistent with the *in situ* analysis, RT-qPCR results revealed that TM3 cell line showed a relatively higher expression of Slfn5os (Figure 2A), which was selected as the cell model for further experiments. The TM3 cells were transfected with empty vector, Slfn5os expression vector, control siRNA (si-NC) or siRNA targeting Slfn5os (si-Slfn5os), and the expression of slfn5 was determined by RT-qPCR. The overexpression of Slfn5os significantly reduced the expression of slfn5 mRNA, while Slfn5os silencing elevated the level of slfn5 mRNA (Figure 2B). These data indicate Slfn5os negatively regulates Slfn5 expression.

Slfn5os knockdown improves TM3 cell proliferation and testosterone secretion

Next we sought to investigate whether Slfn5os is implicated in the functional regulation of Leydig cells. TM3 cells were transfected with si-NC or si- Slfn5os. CCK-8 proliferation assay showed that Slfn5os knockdown enhanced the cell proliferation in TM3 cells (Figure 3A). We also performed Annexin V/PI staining to analyze the apoptotic events upon Slfn5os knockdown. Flow cytometry results revealed that Slfn5os knockdown reduced the percentage of apoptotic events in TM3 cells (Figure 3B). Meanwhile, we also measured the testosterone level

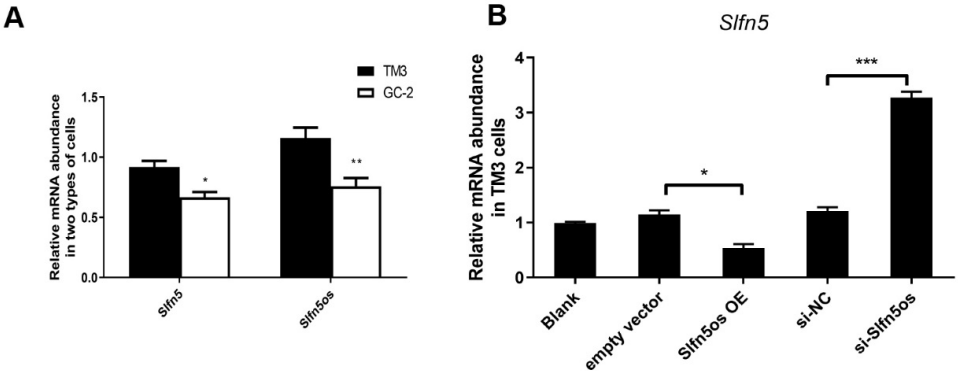

Figure 2: The impact of Slfn5os on Slfn5 expression. (A) The expression levels of Slfn5os on Slfn5were examined by RT-qPCR in TM3 cells (mouse Leydig cell line) and GC-2 cell line (mouse spermatocytes). (B) mRNA expression of Slfn5 in TM3 cells after the transfection of Slfn5os empty vector, Slfn5os expression plasmid, si-NC or si-Slfn5os were determined by RT-qPCR. Data are the summary of three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

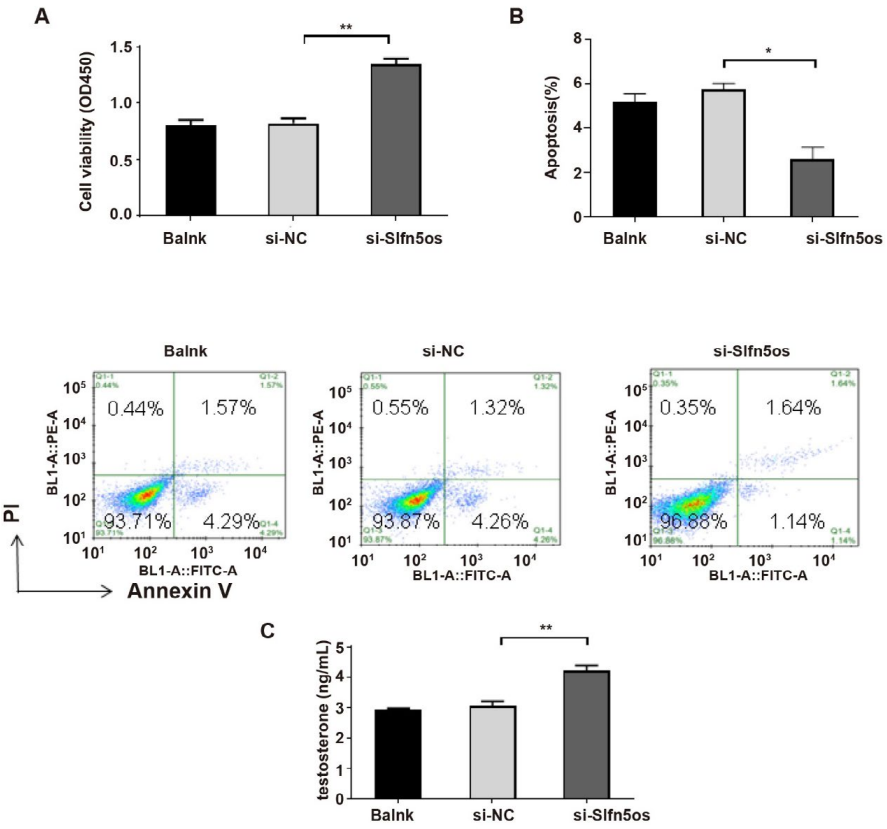

Figure 3: Slfn5os knockdown promotes TM3 cell proliferation, inhibits cell apoptosis and increases testosterone secretion. (A) CCK-8 proliferation assay in TM3 cells transfected with si-NC or si-Slfn5os. (B) Apoptosis events in cells transfected with si-NC or si-Slfn5os were analyzed by flow cytometry. (C) The testosterone concentrations were determined by ELISA in TM3 cells transfected with si-NC or si-Slfn5os. Data are the summary of three independent experiments. * $p < 0.05$; ** $p < 0.01$.

by ELISA, which revealed that testosterone secretion was significantly increased upon Slfn5os silencing (Figure 3C).

Slfn5os overexpression impairs cell proliferation and testosterone secretion

To further corroborate the role of Slfn5os in TM3 cells, we also overexpressed Slfn5os in TM3 cells by transfecting Slfn5os expression plasmid. Cell proliferation assay showed that Slfn5os overexpression suppressed the cell proliferation in TM3 cells (Figure 4A), and apoptosis analysis revealed the induction of apoptosis upon Slfn5os overexpression (Figure 4B). In the meanwhile, testosterone secretion was significantly decreased upon Slfn5os overexpression (Figure 4C). Together, these data suggest that Slfn5os overexpression undermines the cellular function of TM3 cells.

Slfn5os modulates the expression of steroidogenic genes and enzymes in TM3 Leydig cells

To explore the potential mechanisms of Slfn5os-mediated testosterone alteration, RT-qPCR analysis was performed to profile the expression of key genes/enzymes of differentiated Leydig cells from TM3 cells upon Slfn5os knockdown in TM3 cells, including Star, Cyp11α1, Cyp17α1, Hsd3β1, Hsd17β3 and Lhr. As the results showed, the mRNA levels of Star, Cyp11α1, Cyp17α1, Hsd3β1, Hsd17β3 and Lhr were significantly higher in cells transfected with si-Slfn5os when compared to that of the cells transfected with si-NC (Figure 5A). Among these genes, Star, Cyp11α1, Lhr, and Hsd3β1 were increased by 3-7 folds upon Slfn5os knockdown. To further investigate the effects of Slfn5os on the protein levels of LHCGR (Luteinizing Hormone/Chorionic Gonadotropin Receptor), STAR (Steroidogenic Acute Regulatory Protein) and CYP11A1 (Cytochrome P450 Family 11 Subfamily A Member 1), Western blot was performed in TM3 cells upon Slfn5os knockdown or overexpression. Consistent with the mRNA analysis, when Slfn5os gene was silenced the protein levels of STAR, CYP11A1 and LHR were increased; while in the overexpression of Slfn5os, protein levels of STAR and CYP11A1 were significantly decreased (Figure 5B and C). The above results indicate that the expression level of Slfn5os negatively regulates the expression of steroidogenic genes such as Star, Cyp11a1, and Lhr.

Discussion

Non-coding RNAs (ncRNAs) emerge as a new layer of regulation in the reproductive system [22]. microRNAs and Piwi-interacting RNAs have been extensively studied in the post-transcriptional regulation of

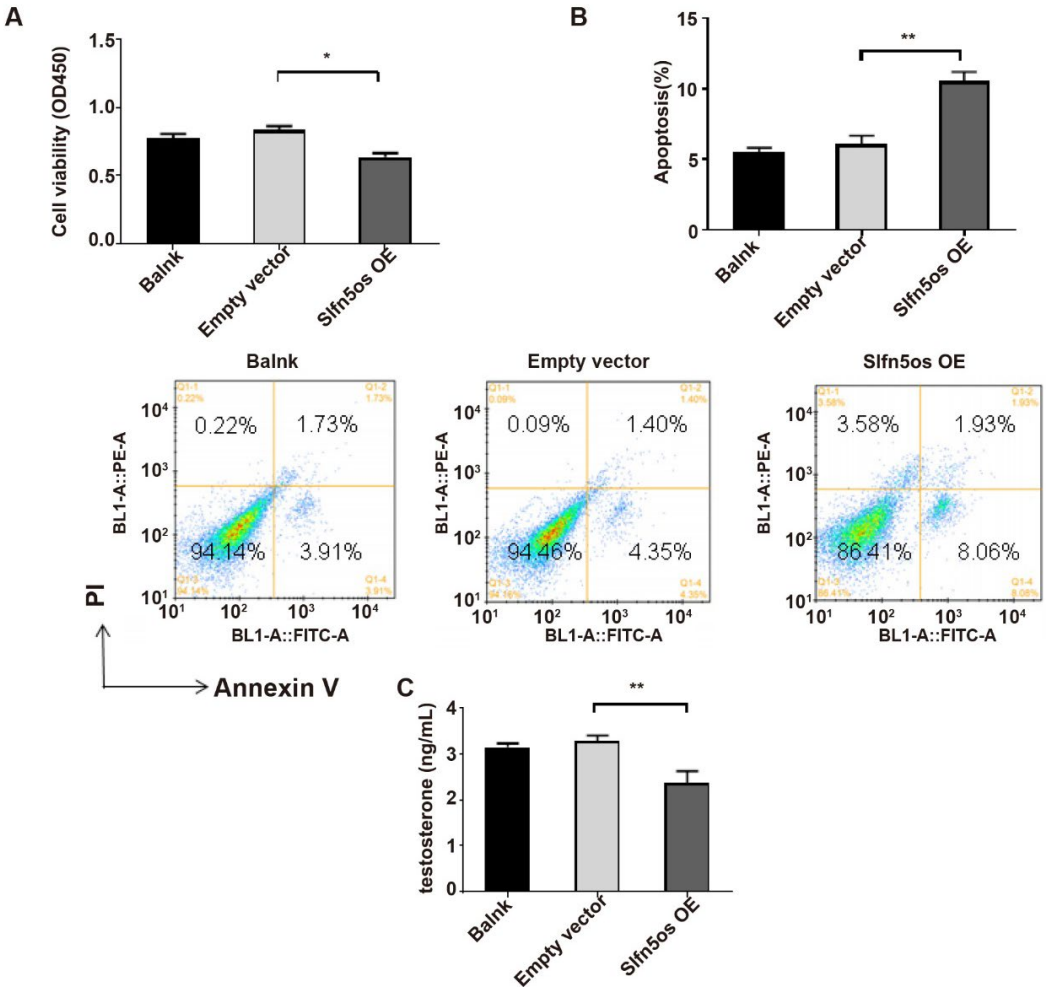

Figure 4: The overexpression of Slfn5os impairs TM3 cell proliferation, induces cell apoptosis and decreases testosterone secretion. (A) CCK-8 proliferation assay in TM3 cells transfected with empty vector or Slfn5os expression vector. (B) Apoptosis events in cells transfected with empty vector or Slfn5os expression vector were analyzed by flow cytometry. (C) The testosterone concentrations were determined by ELISA in TM3 cells transfected with empty vector or Slfn5os expression vector. Data are the summary of three independent experiments. * $p < 0.05$; ** $p < 0.01$.

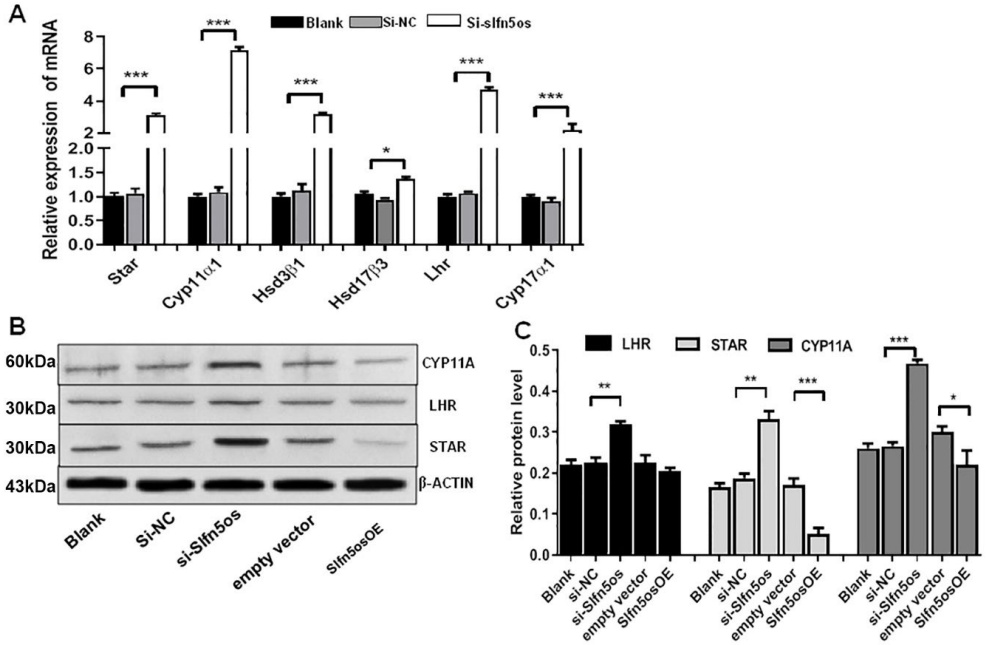

Figure 5: Slfn5os knockdown promotes the expression of steroidogenic genes in TM3 cells. (A) RT-qPCR analysis of the expression of key steroidogenic genes in TM3 cells following the transfection with si-NC or si-Slfn5os. (B) The protein levels of STAR, CYP11A1 and LHR were examined by Western blot in TM3 cells transfected with Slfn5os empty vector, Slfn5os expression plasmid, si-NC or si-Slfn5os. (C) Summary of relative protein levels in (B), the protein level was normalized to β-actin. Data are the summary of three independent experiments. *p < 0.05; **p < 0.01. ***p < 0.001.

mRNAs during spermatogenesis [14, 15]. Recent work also starts to reveal the functions of other ncRNAs in spermatogenesis. For example, circular RNA circ-Bbs9 is implicated in the regulation of Leydig cell proliferation by modulating CyclinD2-dependent pathway [23]. A recent study demonstrated that circular RNAs produced from BOULE locus (circBoule) exerts critical protection against stress-induced infertility by interacting with heat shock proteins, and the production of circBoule is conserved from flies to humans [24]. As another type of abundant ncRNAs, lncRNAs have become another area of intensive research in the reproductive system, and increasing number of lncRNAs has been identified in the testis [25, 26]. In this study, we focused on an uncharacterized lncRNA Slfn5os in the testis of mouse testis. We found that Slfn5os showed testis-specific expression in Leydig cells, and forced Slfn5os reduced testosterone production in TM3 cells. These data suggest its functional role in the regulation of steroidogenesis.

TM3 cells are a widely used cellular model for Leydig cells and steroidogenesis, we therefore studied the functional role of Slfn5os in TM3 cells [27, 28]. Slfn5os is transcribed from the opposite strand of Slfn5 locus (https://www.ncbi.nlm.nih.gov/gene/76392), we then investigated whether Slfn5os regulates the expression of Slfn5 gene. We found that the overexpression of Slfn5os reduced the level of Slfn5 mRNA, while silencing Slfn5os showed the opposite effect. These data indicate that Slfn5os negatively regulates the transcription of Slfn5 locus. However, whether the change of Slfn5 directly affects the function of Leydig cells needs to be further investigated.

We further demonstrated that Slfn5os levels regulate the cell viability and the testosterone production in Leydig cells. A high level of Slfn5os impaired the survival and the secretion of testosterone in Leydig cells. The reduced testosterone level was associated with the downregulation of steroidogenic genes, such as Steroidogenic Acute Regulatory Protein (Star), Cytochrome P450 Family 1 Subfamily A Member 1 (Cyp11α1), Luteinizing Hormone/Choriogonadotropin Receptor (LHR). As those genes play indispensable roles in the production of testosterone [29-31], we speculate that the dysregulation of Slfn5os may affect the level of testosterone production and the spermatogenesis in mouse testis. Since lncRNAs frequently act as molecular sponges for downstream miRNAs [12, 13], we speculate that Slfn5os may affect the expression of steroidogenic genes by targeting miRNAs, which in turns regulates the mRNA levels of steroidogenic genes. Our data also showed that Sfln5os negatively regulates the steroidogenesis in TM3 cells. In the testis, the steroidogenesis is not only controlled by autocrine and paracrine hormones, but can also be regulated by the internal factors [10, 11]. Therefore, it is plausible that Sfln5os expression in Leygid cells may fine-tune the steroidogenesis to prevent the overproduction of steroid hormones.

Our study highlighted a potential function of a testis-enriched lncRNA Slfn5os in regulating the function of Leydig cells. However, the detailed mechanisms how Slfn5os controls the survival and steroidogenesis need to be studied in the future work. A mouse model of Leydig cells-specific deletion or overexpression of Slfn5os will help clarify the functional role of Slfn5os in spermatogenesis. In addition, in is worth investigating whether the dysregulation of Slfn5os is implicated in the malfunction of testis and male infertility.

Conclusion

In summary, we reported a testis-enriched lncRNA Slfn5os in mouse testis, which could regulate the survival and testosterone production in Leydig cells. A high expression level of Slfn5os impairs the survival of Leydig cells, and reduced the production of testosterone as well as the expression of steroidogenic genes. Future work need to focus on the detailed functional roles of Slfn5os in mouse model.

Declarations

Ethics Approval and Consent to Participate

Ethical approval was given by the Ethics Committee of First Hospital of Jilin University.

Consent for Publication

Not applicable

Availability of Data and Material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interest

All the authors declare that they have no conflict of interest.

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Author's Contributions

KM G and HL W contributed to the study conception and design. All authors collected the data and performed the data analysis. All authors contributed to the interpretation of the data and the completion of figures and tables. All authors contributed to the drafting of the article and final approval of the submitted version.

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