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*Original Article*

# miR-4270 regulates cell proliferation and apoptosis in patients with Sertoli cell-only syndrome by targeting GADD45A and inactivating the NOTCH signaling pathway

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**Abstract:** In recent decades, growing data has suggested that microRNAs (miRNAs, miRs) play a critical role in the development of Sertoli cells (SC), including regulating SC maturation, synthesis, proliferation, and apoptosis. Previous reports of miRNA microarray have identified aberrant miR-4270 expression in patients with Sertoli-cell-only syndrome (SCOS). However, it is not known whether miR-4270 is associated with the pathogenesis of SCOS. In this study, we aimed to further investigate the roles and potential mechanisms of miR-4270 on SC proliferation and apoptosis. Our data confirmed that miR-4270 was significantly upregulated in SC of SCOS patients compared with healthy controls. EdU and CCK-8 assays showed silencing of miR-4270 by specific inhibitor significantly enhanced human SC and TM4 cells proliferation. ELISA and flow cytometry assays indicated that miR-4270 knockdown prominently suppressed the apoptosis of human SC and TM4 cells. Furthermore, expression of cell cycle genes, including CCNE1 (cyclin E1), CCND1 (cyclin D1) and CDK4 (cyclin dependent kinase 4), were obviously upregulated in human SC and TM4 cells by qRT-PCR assay after knockdown of miR-4270, while expression of cell apoptotic factors, including CASP3 (caspase 3), CASP6 (caspase 6) and CASP7 (caspase 7), were all markedly decreased. Notably, GADD45A (growth arrest and DNA damage inducible alpha) mRNA was downregulated in SC of SCOS patients, and negatively corrected with miR-4270 expression. Moreover, bioinformatics tools and dual-luciferase reporter assay identified that miR-4270 directly bound the 3'-UTR of GADD45A mRNA to inhibit GADD45A expression. Meanwhile, Western blots analysis validated that the protein expression levels of NOTCH1 (notch receptor 1) and HES1 (hes family bHLH transcription factor 1) were significantly increased in SC and TM4 cells after miR-4270 silencing or GADD45A overexpression. Taken together, our data demonstrated that miR-4270 regulates proliferation and apoptosis in SC of SCOS patients by inactivating NOTCH signaling pathway via GADD45A gene, which may offer a new insight into the development of human SC and provide a promising biomarker for the treatment of SCOS.

**Keywords:** miR-4270, Sertoli cells, proliferation, apoptosis, GADD45A

## Introduction

In recent decades, infertility has been considered a major and widespread public health problem [1]. Approximately 50% of infertile cases may be caused by male factors, including azoospermia, genital tract obstructions, varicocele, cryptorchidism, and testicular failures [2]. Azoospermia is one of the main factors affecting male infertility [3]. Sertoli-cell-only syndrome (SCOS) is a type of azoospermia with a complete loss of male germ cells in the seminiferous tubules [4]. Emerging studies have confirmed that the phenotype of SCOS is associated with genetic variants and chromosome microdeletion [5]. Despite a significant progress in etiology of azoospermia, the pathological mechanism SCOS, remains poorly understood. Spermatogenesis is a complex process including the mitosis of spermatogonia, the meiosis of spermatocytes, and spermiogenesis. Sertoli cells (SC) are localized in the seminiferous epithelium and act as nurse cells to provide the necessary nutrients for the development of dif-

ferent germ cell types [6]. The proliferation of SC is strictly controlled by multiple regulators, such as transcription factors, hormones and non-coding RNAs. Recent studies have shown that SCOS is linked to aberrant proliferation and apoptosis of human SC [7].

MicroRNAs (miRNAs, miRs) belong to a type of small non-coding RNAs with approximately 18-22 nucleotides in length, which are widely detected in different human tissues and negatively regulate the expression of target genes either by targeting mRNAs for degradation or by inhibition of protein translation [8]. In the past years, miRNAs have been reported to participate in a wide range of cellular processes, and their deregulation can lead to diverse diseases including azoospermia [9]. For example, Gao et al. [10] found that miR-10a dependent genetic regulation of meiotic process is crucial for male germ cell development and spermatogenesis in both mouse and human. Yang et al. [7] demonstrated that miR-202-3p controls the cell proliferation, apoptosis, and synthesis function of human SC via targeting LRP6 (LDL receptor related protein 6) and CCND1 (cyclin D1) of the Wnt/β-catenin signaling pathway. Song et al. [11] reported that reduced miR-188-3p expression contributes to apoptosis of spermatogenic cells in patients with azoospermia. In addition, Tang et al. [12] showed that miR-210 is involved in spermatogenesis by targeting IGF2 (insulin like growth factor 2) in male infertility. These findings indicated that miRNAs in SC may influence spermatogenesis. However, roles and regulatory mechanisms of miRNAs in human SC remain largely unclear.

Yao et al. [13] used microarray to analyze the miRNAs profiles in patients with azoospermia and SCOS, and found 174 miRNAs were aberrantly expressed in human SC. Of theses miRs, miR-4270 was shown to significantly upregulate in SCOS compared with azoospermia. miR-4270 is located in human chromosome 3p25.1 and is a human-specific miRNA, which plays an important role in lung adenocarcinoma related-brain metastasis [14]. miR-4270 is lower expressed and has a highly diagnostic value for hepatocellular carcinoma [15]. Recent report has suggested that miR-4270 is relevant to the antigen presentation activity of macrophages [16]. Since miR-4270 expression is increased in SCOS, the roles and potential mechanisms by which miR-4270 regulates proliferation and apoptosis of human SC are well worth determining. Here, we confirmed that miR-4270 was significantly upregulated in SC of SCOS patients compared with healthy controls. GADD45A (growth arrest and DNA damage inducible alpha) was a directly target gene of miR-4270, and miR-4270 regulated proliferation and apoptosis in SC of SCOS patients by inactivating NOTCH signaling pathway via GADD45A gene. The results established a foundation for the further investigation of gene therapy for the treatment of SCOS and for their applications in regenerative medicine.

## Materials and methods

### *Sample collection*

Testicular tissues were obtained from 45 cases of SCOS patients (aged from 19 to 37 years, mean age 26.92 ± 3.14 years) from February 2014 to April 2018 at Peking Union Medical College Hospital (Beijing, China). In addition, testicular samples of 16 healthy controls (aged from 22 to 45 years, mean age 29.30 ± 4.76 years) with normal spermatogenesis who had died in accidents were obtained from January 2008 to December 2016 at Peking Union Medical College Hospital. SCOS patients were diagnosed with physical examination, semen and endocrine examination. Patients with genetic disease, retrograde ejaculation, varicocele, and endocrinological defect were excluded. The healthy controls had fathered at least one child without assisted reproductive measures. Written informed consents were obtained from each participant or their relatives. This experiment was approved by the Institutional Ethics Committee of Peking Union Medical College Hospital, and the activity involving human subjects were carried out under full compliance with the Helsinki Declaration (1991). All samples were stored at -80°C until used for RNA isolation.

### *Cell culture*

The human SC was isolated as previous reported [13]. Briefly, seminiferous tubules were separated from testicular tissues by enzymatic digestion. The human SC and germ cells were obtained from seminiferous tubules by a second enzymatic digestion. After that, cells suspension was seeded into matrigel™ coated

**Table 1.** The specific inhibitors for miR-4270 silencing

| Groups | Specific sequences |
|---|---|
| Inhibitor 1# | 5'-ATAGCTTCAGGGAGTCAGGGG-3' |
| Control 1# | 5'-GGCACTTCACCGGTATAGATCCTT-3' |
| Inhibitor 2# | 5'-GGCAGAAATAGATGGCCTTCCCCT-3' |
| Control 2# | 5'-TAACTATACCTTGCGTCTCACGTA-3' |
| Inhibitor 3# | 5'-CTGCTGGGAAGAAAGTGGGTC-3' |
| Control 3# | 5'-CATAACGTACTAGCGTAAGGAGT-3' |

dishes (BD Biosciences, CA, USA) with DMEM/F12 medium (Gibco, Carlsbad, CA, USA), supplemented with 10% FBS (Gibco, Carlsbad, CA, USA) and incubated at 37°C in 5% $CO_2$ for 24 h. Subsequently, SC attached to culture plates, while the medium containing germ cells were removed. The isolated SC was identified by the exclusion of trypan blue staining and immunnostaining staining with against GATA4, WT1, and SOX9 antibodies. The TM4 cells, a mouse SC cell line, were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured at 37°C in 5% $CO_2$ incubator with DMEM/F12 medium supplemented with 10% FBS containing antibiotic with 1% penicillin and streptomycin (Sigma, St Louis, MO, USA).

*Transfection of inhibitors and plasmids*

The specific inhibitors for targeting miR-4270 were purchased from Hanbio Biotechnology Co., Ltd (Shanghai, China), including inhibitor/control 1#, inhibitor/control 2#, and inhibitor/control 3#. The sequences of each inhibitor/control were shown in **Table 1**. The GADD45A overexpression plasmid for GADD45A upregulation was achieved by GenePharma Co., Ltd (Shanghai, China), and the empty plasmid was used as negative control. Human SC and TM4 cells were cultured with DMEM/F12 medium supplemented and 10% FBS overnight. The cells were transfected with indicated amounts of inhibitor/control and GADD45A overexpression plasmid/empty plasmid by Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA) with Opti-MEM I reduced serum medium (Thermo Fisher Scientific), according to the manufacturer's protocols. After 48 h of cell culture, human SC and TM4 cells were harvested for examining the expression changes of miR-4270 and GADD45A.

*Western blotting analysis*

Human SC and TM4 cells were lysed on ice with RIPA buffer (Thermo Fisher Scientific) with 1% phenylmethanesulfonyl fluoride (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's protocols. Subsequently, cell lysates were precipitated by centrifugation at 12,000 g for 45 min, and the concentration of proteins was measured by a BCA Protein Assay Kit (Millipore, Billerica, MA, USA), according to the manufacturer's instructions. 30 μg of protein from each sample was separated by 8% polyacrylamide gel electrophoresis, and then transferred to a polyvinylidene fluoride membrane (Millipore). After the transfer, the membranes were blocked with 5% nonfat dry milk in TBST for 1 h at room temperature, and incubated overnight at 4°C with anti-NOTCH1 (notch receptor 1) (catalog no: 20687-1-AP; 1:250 dilution; ProteinTech Group, Chicago, IL, USA), anti-HES1 (hes family bHLH transcription factor 1) (catalog no: AV32372; 1:500 dilution; Sigma), anti-GADD45A (catalog no: G3548; 1:500 dilution; Sigma), and anti-ACTB (actin beta) (catalog no: HRP-60008, 1:2500 dilution; ProteinTech Group) antibodies. ACTB was served as loading control. After incubating appropriate second antibodies for 1 h at room temperature, the positive protein signals were observed by Beyoecl Plus Enhanced Chemiluminescence Kit (Beyotime Biotechnology).

*Bioinformatics tools and luciferase reporter assay*

The potential target genes of miR-4270 were predicted by microRNA.org (http://www.microrna.org/microrna/) and TargetScan (http://www.targetscan.org/vert_72/). A fragment from the 3'-UTR of GADD45A mRNA (NM_001924.4) harboring putative miR-4270 binding sites was amplified and inserted into pmirGLO dual-luciferase miRNA target expression vector to get pmirGLO-GADD45A wt plasmid. Similarly, the mutant fragment of 3'-UTR of GADD45A mRNA was subcloned into pmirGLO vector to build pmirGLO-GADD45A mut plasmid. Human SC and TM4 cells were seeded in 24-well cell culture plates at $2 \times 10^5$ cells/well and incubated overnight before transfection. For luciferase reporter assay, cells were cotransfected with 2 μg of pmirGLO-GADD45A wt/mut vector and 100 pmol of inhibitor/control by Lipo-

**Table 2.** Primer sequences for qRT-PCR

| Genes | Primer sequences | |
|---|---|---|
| miR-4270 | F: 5'-TCAGGGAGTCAGGG-3' | R: 5'-TGTCGTGGAGTCGGC-3' |
| U6 | F: 5'-CTCGCTTCGGCAGCACA-3' | R: 5'-AACGCTTCACGAATTTGCGT-3' |
| GADD45A | F: 5'-CCGAAAGGATGGATAAG-3' | R: 5'-CATGTAGCGACTTTCCCGGC-3' |
| CCNE1 | F: 5'-GCCAGCCTTGGGACAAT-3' | R: 5'-CTTGCACGTTGAGTTTGG-3' |
| CCND1 | F: 5'-CGTGGCCTCTAAGATGA-3' | R: 5'-CTGGCATTTTGGAGAGG-3' |
| CDK4 | F: 5'-AGTTCGTGAGGTGGCTT-3' | R: 5'-GGGTGCCTTGTCCAGATA-3' |
| CASP3 | F: 5'-TCTGTTGAAGTTTACAAT-3' | R: 5'-GACAGCCAGTGAGACT-3' |
| CASP6 | F: 5'-AGAATCACTTGAACC-3' | R: 5'-ATTAGACCATAATGCTTC-3' |
| CASP7 | F: 5'-CCTATCCTGCCCTCACAT-3' | R: 5'-CTTCTCCTGCCTCACTG-3' |
| ACTB | F: 5'-TCGTCCACCGCAAATGCT-3' | R: 5'-AACCGACTGCTGTCACCT-3' |

fectamine 3000 transfection reagent according to the manufacturer's protocols. Cell extracts were prepared 48 h after transfection, and luciferase activity was measured by Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) under the SpectraMax M5 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Renilla luciferase activity was used for normalization and served as an internal control for transfection efficiency.

*qRT-PCR assay*

Total RNA from human SC and TM4 cells was isolated by TRIzol® Reagent (Thermo Fisher Scientific), according to the manufacturer's instructions. The cDNA of miR-4270 was synthesized by the All-in-One miRNA First-Strand cDNA Synthesis Kit (GeneCopoeia, Rockville, MD, USA), and cDNA of protein coding genes was performed by reverse transcription using the PrimeScript RT Reagent Kit (Takara, Dalian, China). The primers used for miR-4270, U6, GADD45A, CCNE1 (cyclin E1), CCND1, CDK4 (cyclin dependent kinase 4), CASP3 (caspase 3), CASP6 (caspase 6), CASP7 (caspase 7) and ACTB were designed by Primer Premier 5.0 software. The primers were synthesized from Sangon Biotech (Shanghai, China), and the sequences were shown in **Table 2**. U6 and ACTB were used as internal controls for the miR-4270 and other protein coding genes, respectively. The qPCR condition of miR-4270 were performed in a 20 μl final volume and were done at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 55°C for 30 s. The qPCR procedure for other protein coding genes consisted of an initial step at 98°C for 15 min, followed by 40 cycles of 98°C for 10 s and 60°C for 30 s. At least three independent biological replicates were performed, and the expression level of each gene was normalized to internal control and was calculated with the $2^{-\Delta\Delta Ct}$ method [17].

*Cell apoptosis assay*

Flow cytometry was performed to detected cell apoptosis. Human SC and TM4 cells were seeded at a density of $8 \times 10^5$ cells/well in six-well cell culture plates in DMEM/F-12 supplemented with 10% FBS overnight. After 48 h of cell transfection, $1 \times 10^6$ cells were harvested and washed with cold PBS twice, and incubated with 5 μl propidium iodide (PI) (Sigma) and 5 μl annexin V-FITC (Sigma) for 30 min at room temperature. The apoptotic percentages of cells were analyzed by FACSCalibur Flow Cytometry system (Beckton Dickinson, San Jose, CA, USA). Staining cells simultaneously with Annexin V-FITC (green) and the non-vital dye PI (red) allowed the identification of normal, early apoptotic, late apoptotic and necrotic cells. ELISA assay (enzyme-linked immuno sorbent assay) was also applied to assess cell apoptosis. Cells ($1 \times 10^6$) were collected, washed with cold PBS twice. Then, CASP3 activity was monitored by Cell Death Detection ELISAPLUS photometric enzyme immunoassay kit from Roche (Penzberg, Germany), according to the specifications of the manufacturer.

*Cell proliferation assay*

For EdU assay, human SC and TM4 cells were seeded in 96-well plates with $6 \times 10^3$ cells/well cultured with DMEM/F-12 and 10% FBS overnight to allow the cells to attach. After 24 h of cell transfection, 10 mM of EdU (Sigma) was added and incubated for 24 h. Subsequently, the cells were treated with 0.5% Tritonx-100 (Sigma) in PBS and exposed to 250 μl of Alexa Fluor® 555 (catalog no: ab150078; 1:5000 dilution; Abcam, Cambridge, MA, USA) for 30 min in the dark at room temperature. Nucleus was stained with 2 μl of Hoechst 33258 (ab-228550; 1:5000 dilution; Abcam) for 1 min at

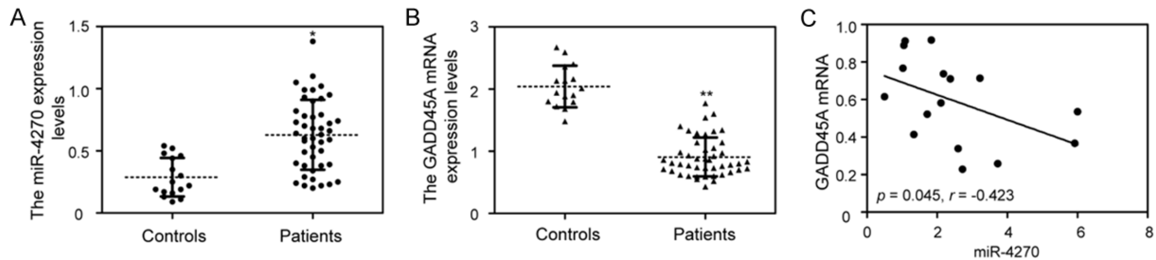


**Figure 1.** The expression levels of miR-4270 and GADD45A mRNA in SC of SCOS patients. A. The relative expression levels of miR-4270 were measured in SC from 45 cases of SCOS patients and 16 cases of healthy controls by qRT-PCR assay. U6 was used as an internal control. B. The lower expression of GADD45A mRNA in SC was observed in SC of SCOS patients compared with healthy controls. ACTB were used as an internal control. C. Spearman's correlation analysis of the relationship between miR-4270 and GADD45A mRNA expression in SC of SCOS patients, and miR-4270 level was inversely correlated with GADD45A mRNA expression. miR: microRNA, SC: Sertoli cells, SCOS: Sertoli-cell-only syndrome, GADD45A: growth arrest and DNA damage inducible alpha, ACTB: actin beta. $^*P < 0.05$, $^{**}P < 0.01$.

room temperature in dark. The percentage of EdU-positive cells was counted by a Leica AF6000 fluorescence microscope (Leica, Bensheim, Germany). For CCK-8 (Cell Counting Kit-8) assay, cells were seeded and transfected in 96-well plates with $6 \times 10^3$ cells/well. The proliferation activity of cells was detected by CCK-8 assay (Dojin Laboratories, Kumamoto, Japan), according to the manufacturer's instructions.

*Statistical analysis*

Statistical analysis was calculated using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). All experiments were repeated at least three times, and the values were presented as the mean ± SD (standard deviation). The data of cell proliferation was assessed by one way analysis of variance with the appropriate post-hoc tests (Dunnet's test or Turkey's multiple comparison). The other data was judged for significant differences by Student's t-test, and the values were considered significant at $^*P < 0.05$, $^{**}P < 0.01$.

## Results

*The expression levels of miR-4270 and GADD45A mRNA in SC of SCOS patients*

miR-4270 expression was detected in SC from 45 cases of SCOS patients and 16 cases of healthy controls by qRT-PCR assay. Consistent with the microarray data, we found that miR-4270 levels were significantly upregulated in SC of SCOS patients compared with healthy controls (**Figure 1A**, $P < 0.05$). Meanwhile, we also quantitatively detected the expression of GADD45A mRNA, and the lower expression of GADD45A mRNA in SC was observed in 45 SCOS patients compared with 16 healthy controls (**Figure 1B**, $P < 0.01$). In addition, we analyzed the relationship between miR-4270 and GADD45A mRNA expression in SC of SCOS patients by Spearman's correlation analysis. As shown in **Figure 1C**, miR-4270 level was inversely correlated with GADD45A mRNA expression ($P < 0.05$). These data indicated that the upregulation of miR-4270 and downregulation of GADD45A mRNA might be associated with the development of SC and were worthy to be further explored.

*The effects of miR-4270 on human SC and TM4 cells proliferation*

Since miR-4270 was markedly upregulated in SC of SCOS patients compared to healthy controls, we then determined the roles of miR-4270 on the proliferation of human SC and TM4 cells by transfection of inhibitor/control #1 - #3. As shown in **Figure 2A**, expression levels of miR-4270 in human SC and TM4 cells transfected with inhibitor #1 exhibited a significant reduce compared with cells transfected with control #1 ($P < 0.01$), reflecting inhibitor #1 could be used in subsequent experiments. Meanwhile, there was no statistical difference between the miR-4270 expression of inhibitor #2 and control #2 or inhibitor #3 and control #3 (**Figure 2B** and **2C**). Subsequently, CCK-8 assay was conducted to evaluate cell proliferation. Compared with the cells treated with control #1, inhibitor #1 treatment significantly pro-

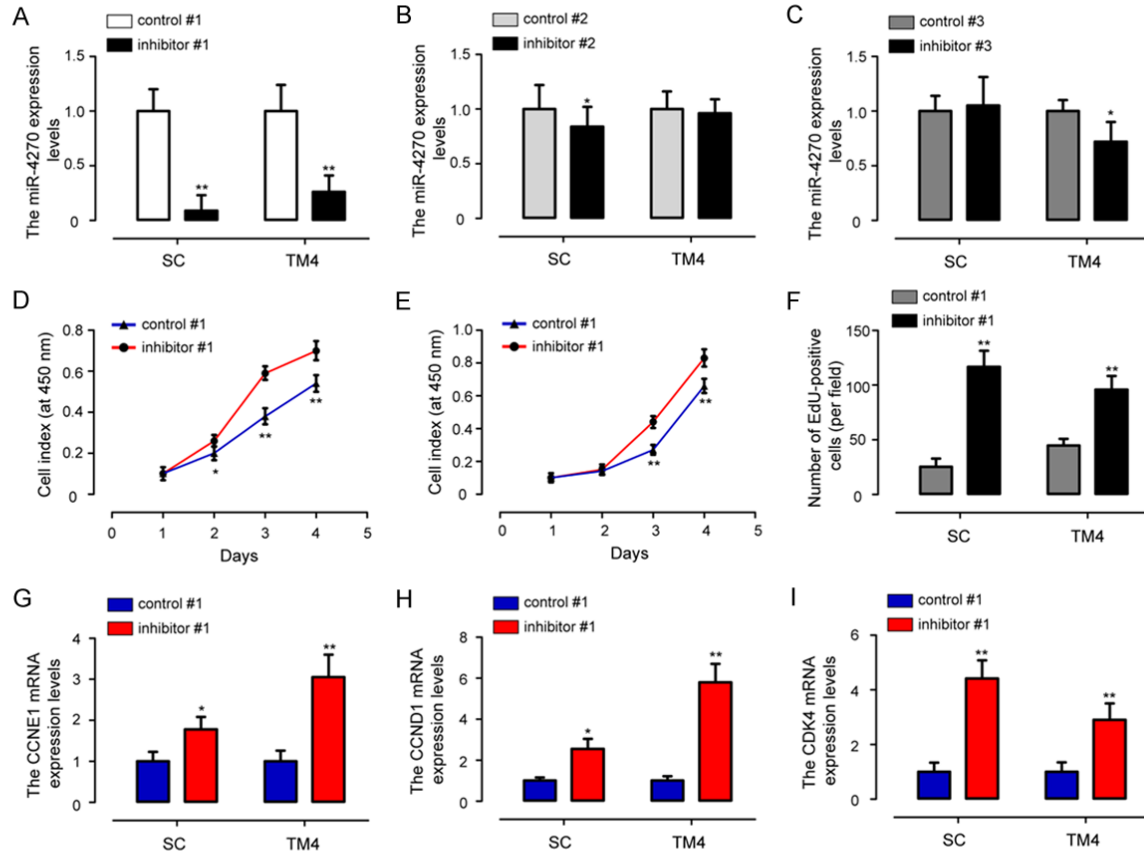


**Figure 2.** The silencing of miR-4270 promoted human SC and TM4 cells proliferation. A. Human SC and TM4 cells were transfected with inhibitor #1 or control #1, and the expression levels of miR-4270 were detected by qRT-PCR assay. B and C. The miR-4270 expression has no statistical difference between inhibitor #2 and control #2 or inhibitor #3 and control #3. D and E. Cell proliferation ability was analyzed 48 h after transfection of inhibitor #1 or control #1 by CCK-8 assay, and found inhibitor #1 treatment significantly promoted the proliferation of human SC and TM4 cells. F. EdU assay revealed that transfection of inhibitor #1 obviously increased the EdU-positive cells compared with cells transfected with control #1. G-I. QRT-PCR analysis of the mRNA expression levels of CCNE1, CCND1 and CDK4 in human SC and TM4 cells by miR-4270 knockdown. CCK-8: Cell Counting Kit-8, CCNE1: cyclin E1, CCND1: cyclin D1, CDK4: cyclin dependent kinase 4. *$P < 0.05$, **$P < 0.01$.

moted the proliferation ability of human SC and TM4 cells (**Figure 2D** and **2E**, $P < 0.05$ or $P < 0.01$). EdU assay showed that transfection of inhibitor #1 obviously increased the EdU-positive cells compared with cells transfected with control #1 (**Figure 2F**, $P < 0.01$), indicating that silencing of miR-4270 enhanced the DNA synthesis of human SC and TM4 cells. Furthermore, qRT-PCR assay revealed that the expression levels of CCNE1, CCND1 and CDK4 were all prominently upregulated by miR-4270 knockdown compared with the control groups (**Figure 2G-I**, $P < 0.05$ or $P < 0.01$). All of these results demonstrated that silencing of miR-4270 promoted human SC and TM4 cells proliferation by regulation of cell cycle progression.

*The roles of miR-4270 on the apoptosis in human SC and TM4 cells*

To further determine the roles of miR-4270 on cell apoptosis, flow cytometry was used to examine the apoptosis rates in human SC and TM4 cells by miR-4270 knockdown. The results indicated that the rates of apoptotic cells in the inhibitor #1 groups were significantly lower than that in the control #1 groups (**Figure 3A**, $P < 0.05$ or $P < 0.01$). Moreover, the apoptotic factors, CASP3, CASP6 and CASP7 were all markedly decreased in inhibitor #1 treated cells than that in control #1 transfected cells (**Figure 3B-D**, $P < 0.05$ or $P < 0.01$). In addition, CASP3 activity in human SC and TM4 cell was

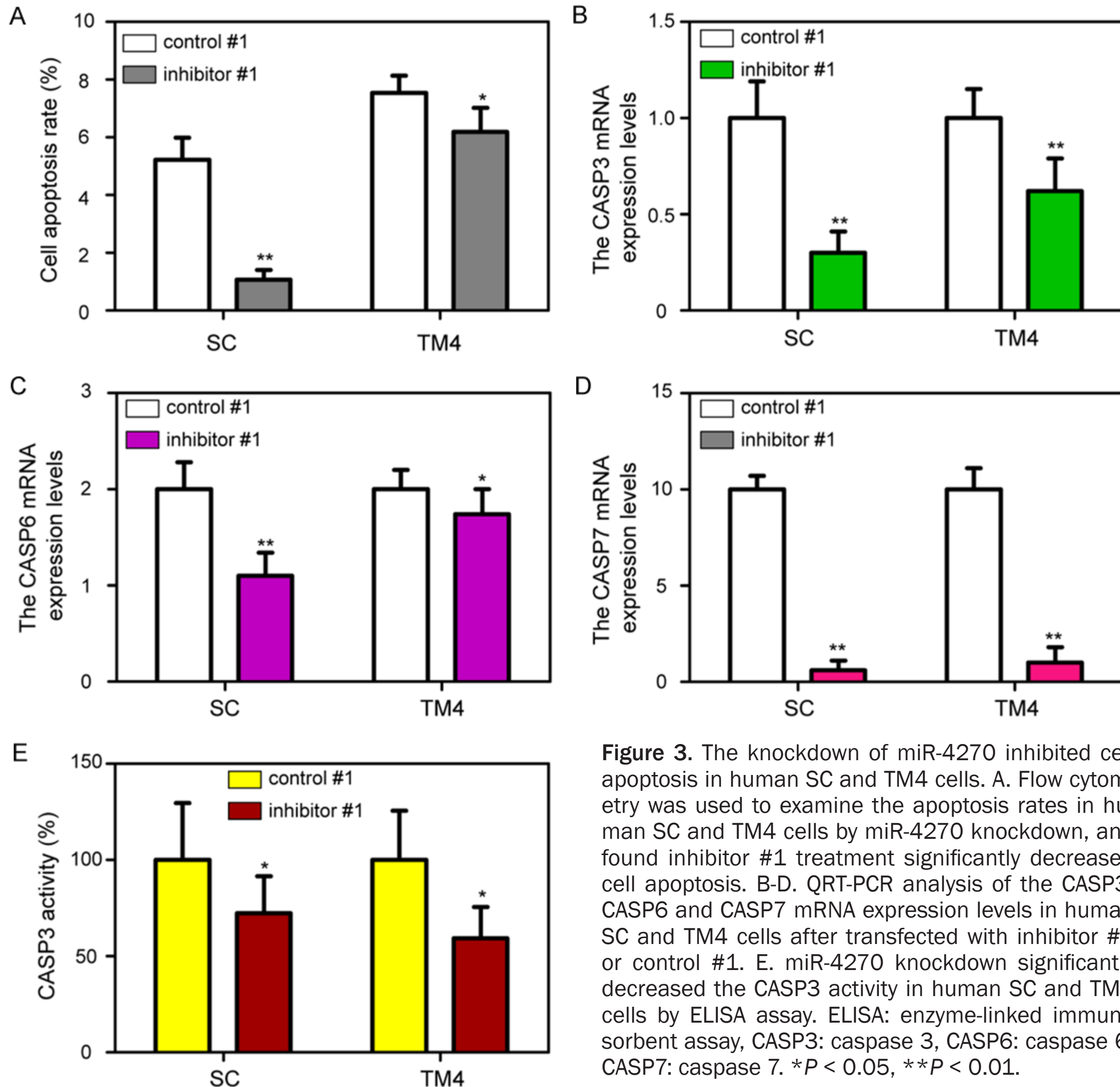


**Figure 3.** The knockdown of miR-4270 inhibited cell apoptosis in human SC and TM4 cells. A. Flow cytometry was used to examine the apoptosis rates in human SC and TM4 cells by miR-4270 knockdown, and found inhibitor #1 treatment significantly decreased cell apoptosis. B-D. QRT-PCR analysis of the CASP3, CASP6 and CASP7 mRNA expression levels in human SC and TM4 cells after transfected with inhibitor #1 or control #1. E. miR-4270 knockdown significantly decreased the CASP3 activity in human SC and TM4 cells by ELISA assay. ELISA: enzyme-linked immuno sorbent assay, CASP3: caspase 3, CASP6: caspase 6, CASP7: caspase 7. *$P < 0.05$, **$P < 0.01$.

also performed by ELISA assay. As shown in **Figure 3E**, the data indicated that CASP3 activity in human SC and TM4 cells transfected with inhibitor #1 were significantly reduced than that in the control cells ($P < 0.05$). The results suggested that miR-4270 knockdown in human SC and TM4 cells inhibited cell apoptosis via the suppression of CASP3, CASP6 and CASP7 production.

*The regulatory mechanism of miR-4270 on GADD45A in human SC and TM4 cells*

To explore the regulatory mechanism of miR-4270 on GADD45A, inhibitor #1 was transfected into human SC and TM4 cells. The results showed that the mRNA and protein expression levels of GADD45A were significantly upregulated by miR-4270 knockdown from 48 h post transfection (**Figure 4A** and **4B**, $P < 0.05$ or $P < 0.01$). Subsequently, we used microRNA.org and TargetScan to predict target genes of miR-4270. Among them, GADD45A was selected because high predictive score and conserved motif "CUCCCUG" that might bind to the "seed region" "GAGGGAC" of miR-4270. The alignment sequences of miR-4270 and the 3'-UTR of GADD45A wt/mut were shown in **Figure 4C**. To validate this prediction, the 3'-UTR of GADD45A wt/mut was inserted into pmirGLO dual-luciferase miRNA target expression vector, and were cotransfected with inhibitor 1# or control 1#. The results showed that luciferase activity in the pmirGLO-GADD45A wt vector

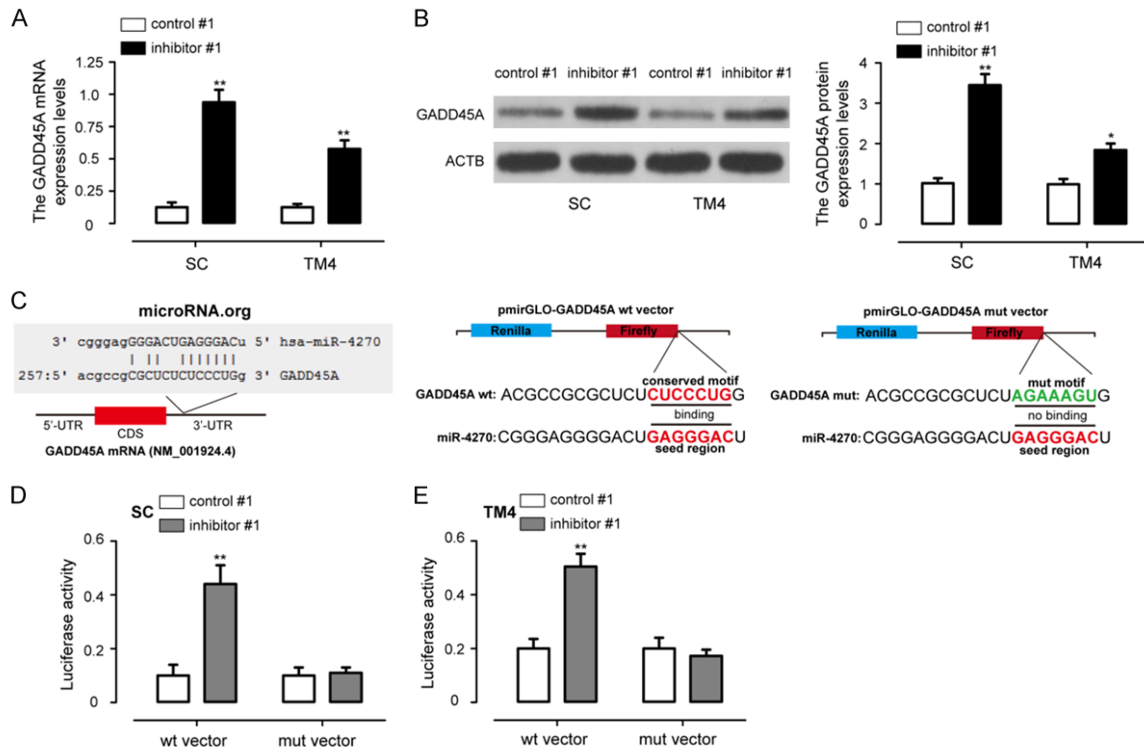

**Figure 4.** GADD45A is a directly target gene of miR-4270. A and B. The mRNA and protein expression levels of GADD45A were significantly upregulated by miR-4270 knockdown from 48 h post transfection. ACTB was served as loading control. C. A putative binding site of miR-4270 in 3'-UTR of GADD45A was predicted by microRNA.org and TargetScan. The alignment sequences of miR-4270 and the 3'-UTR of GADD45A wt/mut. D and E. Human SC and TM4 cells were cotransfected with pmirGLO-GADD45A wt/mut vector and inhibitor/control #1, and luciferase activity was measured. Renilla luciferase activity was served as an internal control for transfection efficiency. The results showed that luciferase activity in the pmirGLO-GADD45A wt vector transfected cells cotransfected with the inhibitor 1# significantly upregulated than that in cells that were transfected with the control 1#. **P* < 0.05, ***P* < 0.01.

transfected human SC and TM4 cells cotransfected with the inhibitor 1# significantly upregulated than that in cells that were cotransfected with the control 1# (**Figure 4D** and **4E**, $P < 0.01$), whereas the pmirGLO-GADD45A mut vector abolished this promotion of luciferase activity, confirming the specificity of the action. These results indicated that miR-4270 directly bound the 3'-UTR of GADD45A to inhibit GADD45A expression.

### *The NOTCH signaling pathway is involved in the effect of miR-4270 on human SC and TM4 cells proliferation and apoptosis*

Studies illustrated that NOTCH signaling pathway is involved in normal spermatogenesis [18]. However, whether the proliferatory and antiapoptotic effects induced by miR-4270 silencing correlated with the NOTCH signaling were unknown. Thus, we measured the expression of NOTCH1 and HES1 by Western blots analysis. The results exhibited that the protein expression levels of NOTCH1 and HES1 were significantly increased in human SC and TM4 cells after miR-4270 silencing (**Figure 5A** and **5B**, $P < 0.05$ or $P < 0.01$). To investigate whether miR-4270 regulating NOTCH signaling expression by suppressing GADD45A, we examined NOTCH1 and HES1 expression in GADD45A overexpression plasmid transfected human SC and TM4 cells. qRT-PCR results confirmed that cells were successfully transfected by GADD45A overexpression plasmid (**Figure 5C**, $P < 0.01$). Mechanically, GADD45A overexpression showed the same results of miR-4270 knockdown (**Figure 5D** and **5E**, $P < 0.05$ or $P < 0.01$). Taken together, these data concluded that miR-4270 targeted NOTCH signaling via GADD45A gene to regulate proliferation and apoptosis in human SC and TM4 cells.

## Discussion

Human SC are the somatic cells of testes that are necessary for normal spermatogenesis by

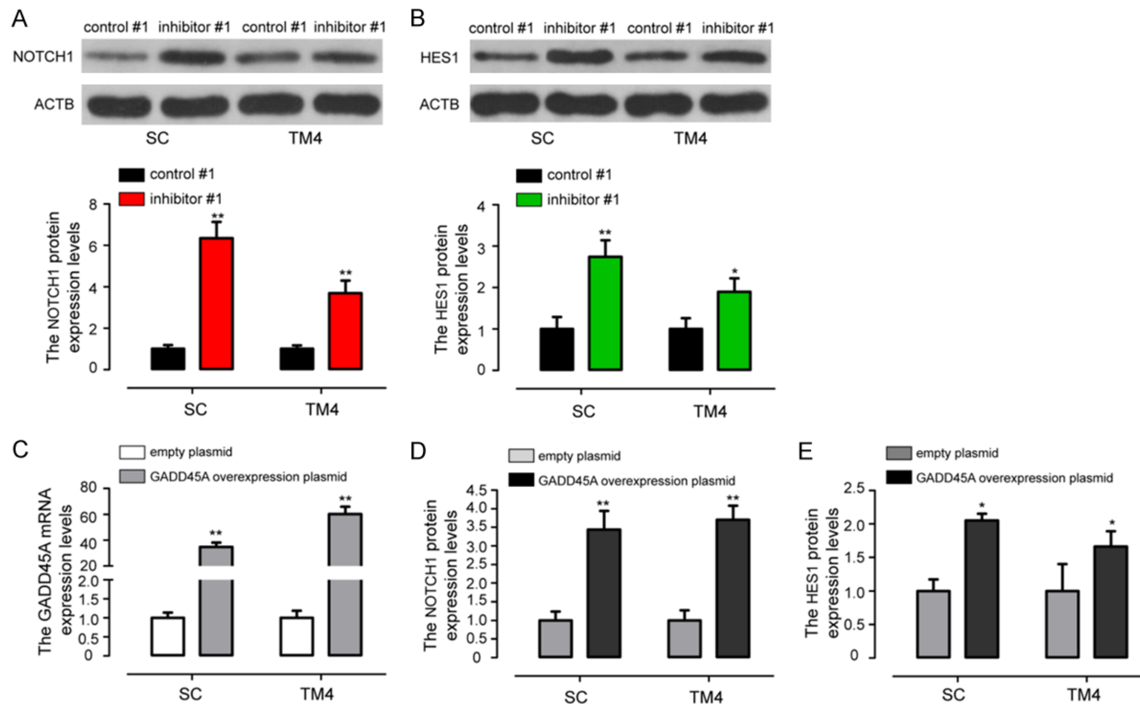


**Figure 5.** miR-4270 regulates NOTCH signaling via GADD45A gene in human SC and TM4 cells. A and B. Western blots analysis of the protein expression levels of NOTCH1 and HES1 in human SC and TM4 cells after transfection of inhibitor #1 or control #1. C. qRT-PCR results confirmed that human SC and TM4 cells were successfully transfected by GADD45A overexpression plasmid. D and E. GADD45A overexpression significantly promoted the protein expression levels of NOTCH1 and HES1 in human SC and TM4 cells, which was similar with the effects of miR-4270 knockdown. NOTCH1: notch receptor 1, HES1: hes family bHLH transcription factor 1. $^{*}P < 0.05$, $^{**}P < 0.01$.

providing a microenvironment within the seminiferous tubules [6]. Several reports have demonstrated that abnormal number and/or roles of SC are tightly linked to male infertility diseases including SCOS [19, 20]. Nowadays, the pathogenesis of SCOS is still elusive. Hence, it is of great significance to examine the pathological mechanism of SCOS, which may provide us new insights into reproductive biology and gene-based therapy for this disease. Although emerging evidence has demonstrated that miRNAs play key roles in regulating SC proliferation and apoptosis [21-24], little is known whether miRNAs could directly regulate the pathogenesis of SCOS. In the present study, we reported that miR-4270 regulates proliferation and apoptosis in SC of SCOS patients by inactivating NOTCH signaling via GADD45A gene. These clues provide *in vitro* loss-of-function evidence of the miR-4270 regulatory mechanism causing SCOS.

Previous studies have mostly focused on miR-4270's roles in various human tumors [14]. For example, miR-4270 is upregulated in breast cancer patients with potential utilization in disease diagnosis and stratification [25]. Recently literature has found that miR-4270 is highly expressed in gastric malignant ascites samples, highlighting its potential as potential prognostic biomarkers of peritoneal metastasis in gastric cancer [26]. Moreover, the microarray-based gene expression analysis also showed that miR-4270 is upregulated in human SC of SCOS patients [13]. However, the roles of miR-4270 on SC are largely unknown. In this study, we confirmed that miR-4270 was significantly upregulated in SC of SCOS patients compared with healthy controls by qRT-PCR assay. Also, we successfully obtained the human SC and TM4 cells models of miR-4270 silencing by transfection of specific inhibitor 1#, which further used to examine cell proliferation and apoptosis. Moreover, our results showed miR-4270 silencing promoted human SC and TM4 cells proliferation by regulation of cell cycle progression, and inhibited cell apoptosis via the suppression of CASP3, CASP6 and CASP7 production. Therefore, these results indicated that

miR-4270 is associated with the proliferation and apoptosis of SC, which may be contributed to explaining the association between miR-4270 and SCOS.

A given miRNAs can regulate target genes by binding 3'-UTR of mRNAs that that participate in various cell biological processes [8]. The GADD45A gene was predicted to be a target gene of miR-4270 by integrated analysis of bioinformatics algorithms. GADD45A is a member of GADD45 family, which is small nuclear and cytoplasmic proteins that bind to other intracellular proteins, such as PCNA, CDK1, and MAP3K4 [27]. GADD45A is implicated in the regulation of cell apoptosis, proliferation, cell cycle, and DNA repair in mammalian cells [28]. Previous study reported that GADD45A is upregulated in spermatogonia during gonocyte differentiation [29]. Here, we identified that GADD45A was negatively regulated by miR-4270. Moreover, luciferase assay with a reporter containing the miR-4270 binding sites from the 3'-UTR of the GADD45A mRNA suggested that miR-4270 suppressed miR-4270 expression by directly binding to its 3'-UTR. Therefore, miR-4270 likely regulates SC proliferation and apoptosis through the inhibition of GADD45A expression.

NOTCH signaling pathway is an evolutionarily highly conserved signaling pathway in mammals, which plays a critical role in cell differentiation and development [30]. Increasing data has demonstrated that NOTCH signaling is involved in normal spermatogenesis [18]. Dysregulation of NOTCH signaling has been found in aryl hydrocarbon receptor (AhR) knockout mice that induced early maturation of spermatocytes and a depletion of primary spermatids [31]. Therefore, we speculated that miR-4270 regulated SC proliferation and apoptosis through the NOTCH signaling. Our results showed that miR-4270 silencing significantly increased the protein expression levels of NOTCH1 and HES1 in human SC and TM4 cells. Interestingly, NOTCH1 and HES1 were dramatically upregulated when GADD45A was overexpressed. The results of the experiment were in agreement with our expectations, indicating that miR-4270 targets NOTCH signaling via GADD45A gene to regulate the pathogenesis of SCOS.

In summary, the study demonstrated that miR-4270 is significantly upregulated in SC of SCOS patients. We highlighted that miR-4270 targets NOTCH signaling pathway via GADD45A gene to regulate proliferation and apoptosis in human SC and TM4 cells. Our study may offer a novel insight into better understanding the underlying mechanism for SCOS and provide a potential target for developing a promising biomarker for treating this disease. Yet understanding the precise mechanisms will require further studies with large-scale samples.

## Disclosure of conflict of interest

None.

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