Original Article
Hypermethylation in promoters of miR-28-5p enhances cell survival in rat ovary granule cells

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Abstract: Polycystic Ovary Syndrome (PCOS) is an epidemic in current modern society, with many reproductive age women suffering from this disease. MicroRNAs have been regarded as genetic factors in the progression of PCOS. The current study aimed to explore the association between methylation of miR-28-5p and PCOS. miR-28-5p expression was first investigated using qPCR in PCOS rats. Methylation levels of miR-28-5p promoter were measured by MSP (Methylation Specific PCR). Rat ovary granule cells were sorted and miR-28-5p expression was measured after cells were treated with 5-Aza (a methylation inhibitor) and miR-28-5p inhibitors. Cell proliferation was detected with CCK8 assay and EdU staining, followed by FCM (flow cytometry) in cells treated with miR-28-5p inhibitors and 5-Aza. Cell apoptosis and cycle distribution were measured. Proteins referring to cell apoptosis and the cell cycle were investigated using Western blotting. Elevated methylation levels of miR-28-5p promoter were confirmed in PCOS rats, with decreased relative miR-28-5p levels. Moreover, 5-Aza attenuated miR-28-5p expression, cell proliferation, and S-phase distribution. However, these trends were reversed by miR-28-5p inhibitors. Present results suggest that miR-28-5p could suppress cell survival in rat ovary granule cells. This research may provide some clues in clinical PCOS therapy based on further in-depth investigation.

Keywords: Polycystic ovary syndrome, methylation, cell proliferation, cell apoptosis, miRNA-28-5p

Introduction

Polycystic ovary syndrome (PCOS) is an epidemic in the modern world, characterized by hyperandrogenism, oligo-ovulation, and metabolic disorders in reproductive age women [1]. Approximately 5-10% of reproductive age women suffer from this lifelong disease. Relative complications include obesity, hyperinsulinemia, and insulin resistance [2]. The etiopathogenesis of PCOS is complex. Environmental and genetic factors have been considered as two potential reasons [3]. Regarding genetic factors, alteration of methylation on promoter of key genes, such as *LINE1*, *EPHX1*, and *CYP-19A1*, has been demonstrated to be regulate the occurrence and development of PCOS [4-6], according to recent reports. Therefore, methylation alteration might be a promising method for PCOS therapy.

DNA methylation has been demonstrated to be an approach of selection expression of a gene.

It is a highly time-specific phenomenon varying with development in the body [7, 8]. Emerging studies have indicated that epigenetic alterations induced by DNA methylation or hypomethylation in promoters may be responsible for metabolic diseases, such as insulin resistance and PCOS [9, 10]. Aberrant DNA methylation of certain genes has been demonstrated to be associated with PCOS. A recent study addressed by Wang et al. found that upregulated expression of LHCGR (LH/choriogonadotropin receptor) induced by decreased LHCGR methylation status might be a potential mechanism causing PCOS [11]. Aberrant DNA methylation of transposons, such as LINE1/L1 (long interspersed nucleotide element-1) has been confirmed to be a factor reflecting disease states [4, 12]. Moreover, PPARGC1A, EPHX1, and CYP19A1 have been indicated as plausible genes associated with PCOS [5, 6, 13].

MicroRNAs, characterized as a single-stranded non-coding RNA sequences (19-25 nucleoti-

des), have been widely research in many disorders. This type of RNA has been regarded as a pivotal factor related to reproductive disorders, including endometriosis, poor ovarian response, and PCOS [6, 14, 15]. Based on miRNA arrays, Huang et al. investigated expression profiles of miRNAs on normal and PCOS cumulus cells isolated from one patient [16]. Furthermore, miR-509-3p has been screened out to as a key miRNA between normal and PCOS cells. Results have indicated that miR-509-3p can positively regulate secretion of oestradiol via targeting MAP3K8. Moreover, several miRNAs have been demonstrated to play essential roles in mediating occurrence and development of PCOS [2, 17, 18]. Many studies have revealed miR-28-5p as an oncomiR through enhancing cell proliferation activity, inhibiting cell apoptosis, re-distributing cell cycle, and promoting cell migration and invasion ability, as well as other malignant activities in several cancers [19-21]. Lower apoptotic rates and higher proliferation rates have been demonstrated to be risky factors for PCOS patients [22]. However, the mechanisms require further investigation. The current study provides the first reference suggesting that methylation of miR-28-5p promoter mediates PCOS status in regulating cell apoptotic rates and proliferation.

In this study, expression of miR-28-5p, along with its promoter methylation status, was investigated. A methylation inhibitor, 5-Aza, and miR-28-5p inhibitors were included in these experiments, demonstrating that expression of miR-28-5p regulated and mediated by miR-28-5p promoter's methylation may play a role in cell proliferation and apoptosis in rat ovary granulosa cells. Results suggest that epigenetic regulation may play a key role in cell survival of rat ovary granule cells, providing some clues concerning occurrence and development.

Materials and methods

Cell isolation, culture, and treatment

Rat ovary granulosa cells were provided by Procell Bio (CP-R050, Procell Bio, Wuhan, China). Briefly, female Sprague Dawley rats underwent anesthesia with pentobarbital. The rats were then sacrificed. Subsequently, ovaries were obtained after washing with precooled PBS. Afterward, the ovaries were placed in a culture dish containing DMEM/F12 culture solution. Follicles were then pierced to release ovary granulosa cells, followed by trypsin digestion for 60 minutes at 37°C. Furthermore, fetal calf serum was added to stop digestion and the supernatant was kept after centrifugation (800 rpm for 5 minutes). Finally, the cells were cultured in the following media: DMEM/F12 (HyClone, USA), 15% FBS (GBICO, USA), EGF (10 ng/mL), 100 U/mL Penicillin, and 100 U/mL Streptomycin.

DNA methyltransferase inhibitor 5-Aza was provided by Sigma (A3656, USA). It was resolved in DMSO. A final concentration at 0.25 μM was set for further cell treatment. Next, the miR-28-5p inhibitor, designed and synthesized by GenePharma (Suzhou, Jiangsu, China), was transfected into cells using Lipofectamine 2000 Transfection Reagent (provided by Thermo Fisher Scientific USA). Cells were pre-treated for 24 hours for further detection. A nonsense sequence was used as the negative control.

PCOS rats and tissue collection

Female adult Wistar rats (110±20 g) were purchased from the Experimental Animal Center of Southern Medical University. Rats with about 3 estrous cycle periods within two weeks were used. Rats meeting selection standards were divided into two groups (Normal vs. PCOS groups, n=6). Rats in the PCOS group received pellets containing DHT using hypodermic injections (45 mg/Kg) to induce polycystic ovary syndrome. All procedures were performed according to Guidelines for the Care and Use of Laboratory Animals. This study was approved by Nanfang Hospital, Southern Medical University.

qPCR

Total RNA was obtained from approximately 50 mg tissues or 10^6 cells using TRIzol (TAKARA, Japan). Next, 400 ng of total RNA was reversed into cDNA using Bestar qPCR RT kit (2220#, DBI Bio, Germany), according to instructions. For miRNA, a miRNA RT kit was used, according to instructions, to perform reverse transcription. U6 small nuclear RNA (Takara) was regarded as an internal reference for miR28-5p. Expression of miRNA/mRNA was quantified by Stratagene Real time PCR machine (Mx3000P, Agilent, USA) using SYBR Green qPCR kit (DBI Bio, Germany). Fold changes were analyzed using the $2^{-\Delta\Delta CT}$ formula. All qPCR reactions were conducted in triplicate. Primers used in these experiments were designed using Primer

Table 1. Primers used in real-time qPCR

Name	Sequence (5'-3')
GAPDH F	CCTCGTCTCATAGACAAGATGGT
GAPDH R	GGGTAGAGTCATACTGGAACATG
PROK1 F	CAACTGTCTCTGACTGTGCG
PROK1 R	AAGGGATCTTGTGGCTTCCA
U6 F	CTCGCTTCGGCAGCACA
U6 R	AACGCTTCACGAATTTGCGT
Mir-28-5p F	ACACTCCAGCTGGGAAGGAGCTCACAGTCTA
Mir-28-5p R	CTCAACTGGTGTCGTGGA

F: Forward primer(s); R: Reversed primer(s); U6: internal reference for miRNA.

5.0 (Canada) (listed in **Table 1**) and synthesized by Sangon Bio (Sangon Bio, Shanghai, China).

Western blot

Total protein was extracted after tissues were minced. This was followed by protein lysates digestion. Total protein concentrations were quantified using the BCA method (#23227, Thermo, USA), according to instructions. Afterward, SDS-PAGE was conducted. Proteins were electrophoretically transferred to polyvinylidene difluoride membranes (IPVH00010, Millipore). Primary antibodies of caspase 3, bcl-2, bax, cyclin D1, and p62, with dilution at 1:500 or GAPDH (ab8245, Abcam, USA), were provided from ABCAM. They were used to immunize against rat proteins, respectively. A rabbit anti-mouse secondary antibody (ab6728, Abcam, USA) was then used. Subsequently, a Western chemiluminescent ECL kit (Millipore) was used to detect bound antibodies. Intensities of labeled proteins were visualized using X-film (SUPER RX-N-C, Juxing Com, Guangxi, China).

Methylation specific PCR (MSP)

Genomic DNA was purified from tissues and cells were treated using a DNA extract Kit (BSC05S1, Boier Bio, Hangzhou, China). They were collected for further sodium bisulfite treatment using TaKaRa EpiTaq HS (TAKARA), according to manufacturer instructions. Sequences of methylated or unmethylated specific primers for further PCR reactions were as follows:

Methylated: F, TTATGTTTTTTGTTGGTTAAGTCGT, R, AACTACACTCTAATACAACCCTCGC, and Unmethylated: F, TTATGTTTTTTGTTGGTTAAGTTGT, R, ACTACACTCTAATACAACCCTCACT. Gel electrophoresis was used for the products of PCR.

Flow cytometry

Pre-treated cells were collected and re-suspended at a density of 10^4 cells per test. Cell apoptosis was detected using BD FCM Calibur™ flow cytometry (BD Bioscience). This was followed by Annexin V-FITC and PI staining (BD Bioscience). Cells marked as Annexin V positive and PI negative were accounted as cell death. For cell cycle distribution assays, cells were staining PI.

CCK8

A CCK8 assay kit (Beyotime, Shanghai, China) was used to investigate cell proliferation activity, according to manufacturer instructions. Briefly, 10^4 cells, pretreated for 48 hours, were incubated for 4 hours using the CCK8 solution. Subsequently, the supernatant was obtained for microplate spectrophotometer detection (Bio-Rad Labs, Sunnyvale, CA) at 450 nm.

EdU staining and FCM

Cells, pre-treated for 24 hours, were collected and underwent EdU staining, according to manufacturer instructions. Afterward, cells were placed into flow tubes for fixation and transparentation with 4% paraformaldehyde and 0.5% TritonX-100, respectively. Apollo staining solution was added into tubes followed by re-transparentation and resuspension with PBS. Finally, cells were subjected to BD FCM Calibur™ flow cytometry (BD Bioscience) for FCM detection.

Statistical analysis

All data are presented as mean ± SD. Student's t-test or one-way analysis of variance, followed by Turkey's test, were used to investigate differences between the groups. Statistical analysis was performed with SPSS 16.0 software for windows (IBM SPSS Inc, USA). *P*-values less than 0.05 indicate significant differences. All experiments were performed in triplicate.

Results

Expression of miR-28-5p and methylation levels of miR-28-5p promoters in PCOS rats

To investigate expression of miR-28-5p in 6 pairs of tissues, qPCR was used. According to **Figure 1A**, miR-28-5p was downregulated significantly in tissues from PCOS patients. Fur-
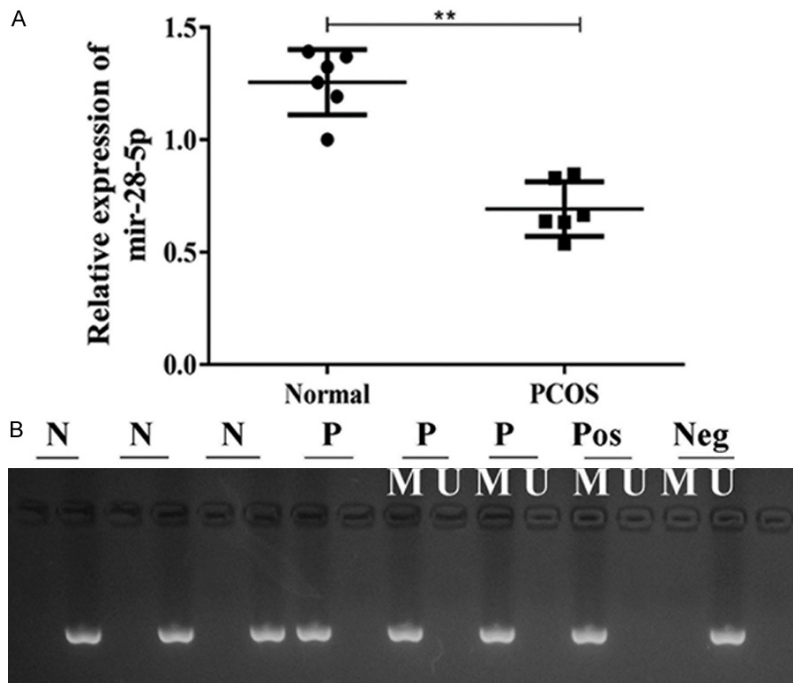
Figure 1. Investigation of miR-28-5p expression in PCOS and normal. A. miR-28-5p expression was significantly downregulated in PCOS; B. Hypermethylation of miR-28-5p promoter was detected using the MSP method. N, Normal; P, PCOS; Pos, Positive test; Neg, Negative test, M, methylation primer, U, Unmethylation primer. **p < 0.01 vs. Normal.
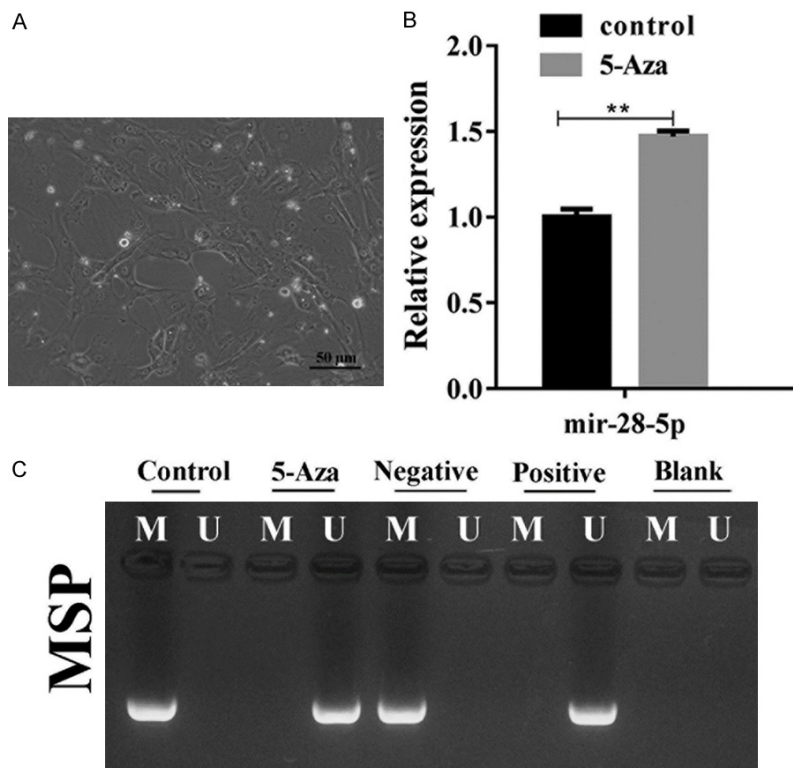

Figure 2. Detection of methylation levels of miR-28-5p in rat ovary granule cells treated with 5-Aza. A. Rat ovary granule cells bright field (200 magnification); B. 5-Aza regulated miR-28-5p; C. Represents 5-Aza demethylated miR-28-5p promoter. **p < 0.01.

thermore, this study determined whether methylation levels of miR-28-5p promoters were dysregulated using the MSP method. Results, as shown in **Figure 1B**, suggest that methylation levels in promoters of miR-28-5p were abnormal in tissues from PCOS patients (P), compared with normal patients (N). Results indicate that aberrant methylation levels in promoters of miR-28-5p might induce downregulation of miR-28-5p.

Expression of miR-28-5p and methylation levels of miR-28-5p promoters meditated by 5-Aza

To further validate promoter methylation as a mediator of miR-28-5p expression, 5-Aza was used. In this section, rat ovary granule primary cells (**Figure 2A**) were subjected to treatment with 5-Aza. Expression of miR-28-5p was analyzed by qPCR. As anticipated, expression of miR-28-5p in cells treated with 5-Aza presented relatively higher expression levels (**Figure 2B**). Finally, MSP assays were performed to confirm alteration of methylation levels of miR-28-5p promoters (**Figure 2C**). Taken together, blocking of methylation levels induced by 5-Aza exerted positive regulation of miR-28-5p expression.

Alteration of methylation levels of miR-28-5p meditated cell proliferation

To explore the biological effects of miR-28-5p on cell proliferation activity, a series of functional restoration assays were conducted. CCK8 assay was perform-
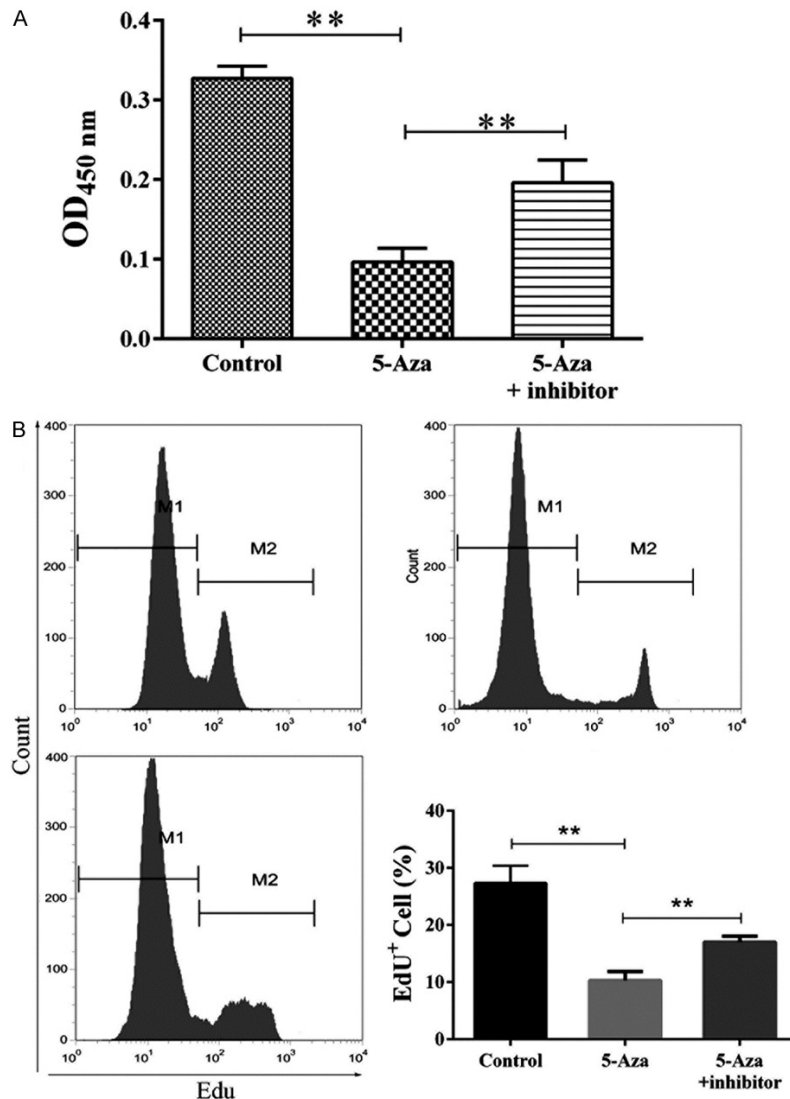
Figure 3. Cell proliferation activity in ovary granule cells. A. CCK8 assay was used to measure cell proliferation at OD 450 after cells were treated with 5-Aza or/and miR-28-5p inhibitor; B. EdU staining was used to measure cell proliferation and analyzed by FCM after cells were treated with 5-Aza or/and miR-28-5p inhibitor. **p < 0.01.

Western blot were performed. Overexpression of miR-28-5p induced by 5-Aza significantly promoted apoptosis rates. In contrast, knockdown by miR-28-5p inhibitor transfection presented a protective role to rat ovary granule cells (**Figure 4A**). Hence, this study further investigated cell cycle distribution based on the above treatment. Subsequently, it was found that 5-Aza promotes G1-phase distribution and blocks the S-phase. However, miR-28-5p inhibitors reversed 5-Aza-induced G1-phase enhancement and S-phase inhibition (**Figure 4B**). Finally, this study found that Caspase 3 and Bax expression levels were elevated by 5-Aza, significantly. However, expression was then suppressed by miR-28-5p inhibitors. Additionally, Bcl-2 and Cyclin D1 expression was inhibited by the methylation inhibitor, 5-Aza. Expression was significantly reversed, however, by miR-28-5p inhibitors (**Figure 4C**).

Present results indicate that upregulation of miR-28-5p induced by 5-Aza diminished methylation levels of miR-28-5p promoters, promoted cell survival, and inhibited cell apoptosis via redistributing the cell cycle.

ed. According to **Figure 3A**, cell proliferation was attenuated after cells were treated with 5-Aza. This diminished condition was then rescued by miR-28-5p inhibitor transfection (**Figure 3A**). Similar results were found in cells using EdU staining post FCM (**Figure 3B**).

Alteration of methylation levels of miR-28-5p meditated cell apoptosis and cell cycle distribution

To confirm the function of miR-28-5p on cell apoptosis and cell cycle distribution, FASC and

Discussion

Polycystic ovary syndrome (PCOS) has a high prevalence worldwide. It has been associated with disorders of endocrine condition, characterized by hyperandrogenism, menstrual disturbances, and polycystic ovaries [1]. Recent studies have discovered a strong positive association between PCOS and other sequelae, including breast cancer [23]. Evidence for the genetic
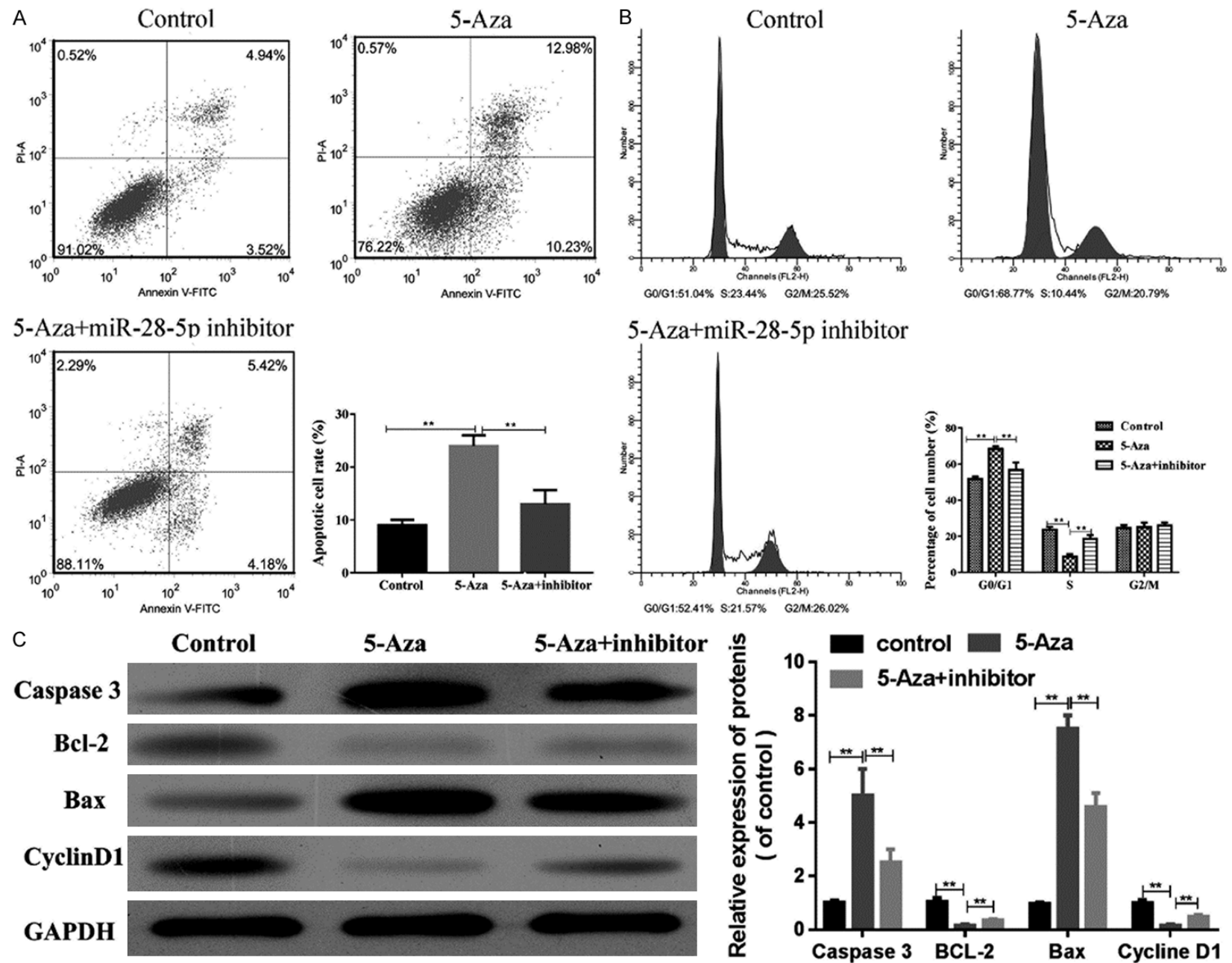
miR-28-5p in OG cells

Figure 4. Cell apoptosis and cycle distribution was investigated. Cell apoptosis (A) and cell cycle distribution (B) were explored using Annexin V-FITC/PI and PI staining after rat ovary granule cells were treated with 5-Aza or/and miR-28-5p inhibitors; (C) Proteins related to apoptosis (Caspase 3, Bax, Bcl-2) and cell cycle mediation (cyclin D1) were measured using Western blot. **p < 0.01.

basis of PCOS is very broad. The pathophysiology of PCOS has not been adequately interpreted. However, environmental and genetic factors have been regarded as two potential switches mediating PCOS occurrence and development [3]. Emerging evidence has suggested that alteration of methylation plays a key role in PCOS [6, 24]. The current study confirmed expression differences in miR-28-5p between tissues from normal and PCOS rats. Further results confirmed that aberrant methylation of miR-28-5p can silence its expression, promoting ovary granule cell proliferation. Present findings demonstrate that gene expression mediatio by DNA methylation plays a pivotal role in PCOS.

Aberrant cell survival in PCOS granulosa cells has been demonstrated in many studies [25, 26]. However, the underlying mechanisms are not well understood. Chen et al. suggested that CFTR is downregulated in granulosa cells of PCOS rats, with suppressed PCNA expression, a pro-proliferation gene [26]. miR-145 has been found to be suppressed in ovarian granulosa cells from PCOS patients. Further exploration has demonstrated that miR-145 can inhibit cell proliferation by targeting IRS1 via MAPK/ERK signaling pathways [27]. A recent study indicated that miR-182 is downregulated in PCOS rats and, therefore, lower apoptotic rates are mediated by miR-182 through targeting Smad7 [28]. miR320 and miR-383 have been reported to mediate cell proliferation and apoptosis in granulosa cells through targeting E2F1 and SF-1 [29]. This study confirmed that cell proliferation was enhanced in ovarian granulosa cells from PCOS rats. However, cell apoptosis was suppressed.

miR-28-5p has been documented as a potential plasma marker for diagnosis of pulmonary embolisms [30]. A clinical study showed that miR-28-5p was upregulated in esophageal cancer tissues, compared with para-cancerous normal tissues [31]. It has been demonstrated to be a mediator in colorectal cancer, as well [19]. However, there are no studies referring to miR-28-5p and PCOS. MicroRNAs have been defined as a critical regulator in PCOS, accord-

ing to several studies. miR-93 has been proven to a promoter of cell proliferation in granulosa cells [15]. The present study found that miR-28-5p expression was downregulated in PCOS, compared with normal tissues. Knockdown of its expression enhanced cell proliferation, blocked cell apoptosis, and re-distributed the cell cycle. Present results indicate that miR-28-5p plays a key role in PCOS pathogenesis.

DNA methylation dysregulation of genes is related to various physiological processes, including blood pressure and cellular cholesterol homoeostasis, obesity, insulin, and type II diabetes. These have been widely associated with PCOS pathogenesis [10, 32]. PPARGC1A, playing an important role in type II diabetes, has recently been illustrated to be a candidate potential biomarker for PCOS [13, 32]. Studies have suggested that aberrant DNA methylation of its promoter scan mediate PCOS pathogenesis through regulation of mtDNA content [13]. CYP19A1 is a critical gene in estrogen biosynthesis. Stueve et al. explored CYP19A1 promoter methylation levels in saliva in urban girls, with results indicating that epigenetic marks in surrogate tissues may improve risk prediction [6]. According to a report from Sang et al., methylation of the EPHX1 promoter is associated with PCOS [5]. However, there is limited evidence referring to methylation alteration in miRNAs. In the present study, miR-28-5p promoter hypermethylation in PCOS was proven to be a critical mediator regulating miR-28-5p expression.

Conclusion

In summary, the current study demonstrated that miR-28-5p was decreased in PCOS. This downregulation was identified to be the hypermethylation of miR-28-5p promoters. Moreover, miR-28-5p might act as a protector in PCOS pathogenesis via inhibiting ovary granule cell proliferation, thus promoting cell apoptosis. This study provides the first reference between miR-28-5p and PCOS. However, in-depth mechanisms of miR-28-5p in PCOS pathogenesis require further exploration.

Disclosure of conflict of interest

None.

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