CDKN2B-AS1 is overexpressed in polycystic ovary syndrome and sponges miR-181a to promote granulosa cell proliferation

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MiR-181a suppresses the proliferation of mouse granulosa cells, which participate in polycystic ovary syndrome (PCOS), suggesting the potential role of miR-181a in PCOS. Our bioinformatics analysis revealed that miR-181a could bind CDKN2B-AS1, a lncRNA regulates ovarian endometriosis. This research was, therefore, conducted to explore the potential crosstalk between CDKN2B-AS1 and miR-181a in PCOS. Expression analysis of CDKN2B-AS1 and miR-181a in follicular fluid from 60 PCOS patients and 60 controls was done with reverse transcriptions-quantitative PCRs. The direct interaction between CDKN2B-AS1 and miR-181a was predicted by IntaRNA and confirmed by RNA pull-down assay. CDKN2B-AS1 in nuclear and cytoplasm of granulosa cells was detected by cellular fractionation assay. The role of CDKN2B-AS1 and miR-181a in granulosa cell proliferation was analyzed by 5-bromodeoxyuridinc assay. In this study, CDKN2B-AS1 was expressed in high amounts in PCOS, whereas miR-181a was downregulated in PCOS, CDKN2B-AS1 was detected in both nucleus and cytoplasm. Although CDKN2B-AS1 and miR-181a were not closely correlated, CDKN2B-AS1 directly interacted with miR-181a. CDKN2B-AS1 and miR-181a overexpression failed to affect the expression of each other. In addition, the inhibitory effect of miR-181a on granulosa cell proliferation was attenuated by CDKN2B-AS1. CDKN2B-AS1 is overexpressed in PCOS and may sponge miR-181a to promote granulosa cell proliferation. Our study characterized a novel CDKN2B-AS1/miR-181a pathway in PCOS. This novel pathway may serve as a potential target to treat PCOS. *Anti-Cancer Drugs* 34: 207–213 Copyright © 2022 The Author(s). Published by Wolters Kluwer Health, Inc.

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Introduction

Polycystic ovary syndrome (PCOS) is a common clinical disorder that affects women at reproductive age, and it is characterized by the altered production of androgens and secretion of male sex hormones in rare cases [1,2]. As the most common cause of failures in pregnancy, PCOS affects about 27% of women at their childbearing age [3,4]. Without proper treatment, PCOS in long-term may increase the risk of a series of health problems, such as heart disease, acne scars, or even cancers [5,6] No cure is available for PCOS, current therapeutic approaches mainly focus on controlling symptoms, whereas symptoms vary a lot across patients, which is a big challenge in the treatment of PCOS [7].

Previous studies have shown that genetic and molecular factors are critical contributors to the progression of PCOS in many critical pathological processes, such as insulin resistance and ovarian dysfunction [8–11]. These studies also indicate that regulating the expression or activation of certain critical molecular players in PCOS, such as transforming growth factor-β and neuronal androgen receptor, may contribute to the treatment of this clinical disorder [10,11]. Therefore, molecular targets are always needed to screen for the ones with high safety. LncRNAs and miRNAs regulate nearly all human diseases, such as PCOS, by regulating gene expression [12,13], indicating the potential roles of these RNAs in the treatment of PCOS. Specifically, miRNAs suppress the production of proteins with critical functions in PCOS as translational and transcriptional levels to regulate PCOS [13]. In contrast, lncRNAs may serve as the targets, or sponges (endogenous competing RNAs) of miRNAs to be involved in the pathological changes of PCOS [12,13]. In a recent study, miR-181a was reported to suppress the proliferation of granulosa cells [14]. It has been well established that abnormal proliferation of granulosa cells can promote the initation and progression of PCOS [14], suggesting the potential role of miR-181a

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in PCOS. Our bioinformatics analysis revealed that miR-181a could bind to CDKN2B-AS1, a lncRNA regulates ovarian endometriosis [15]. Therefore, it will be reasonable to hypothesize that CDKN2B-AS1 and miR-181a may interact with each other to participate in PCOS. This study was, therefore, carried out to study the crosstalk between CDKN2B-AS1 and miR-181a in PCOS.

Materials and methods

Collection of samples from both polycystic ovary syndrome patients and controls

Follicular fluid was donated by both PCOS patients (n = 60; 27.1 ± 3.4 years) and controls (n = 60; 27.0 ± 3.7 years). All these participants were admitted to Suzhou Hospital of Integrated Traditional Chinese and Western Medicine between March 2019 and March 2021 (Ethics approval was obtained from this hospital). The diagnosis of PCOS was performed through blood analysis and pelvic ultrasound. Inclusion criteria: (a) patients diagnosed for the first time; (b) patients with no blood relationship. Exclusion criteria: (a) patients with initiated therapy; (b) patients complicated with other ovary diseases or severe disorders in other organs; and (c) patients with severe infections. Control groups included females who received IVF or intracytoplasmic sperm injection due to male factor or IVF. Informed consent was provided by all participants. Clinical data of participants was presented in Table 1.

Cells, cell culture, and transfections

Human granulosa cells (Procell Life Science & Technology Co., Ltd., Wuhan, China) were cultivated in an incubator with humidity, temperature, and humidity set to 95%, 37 °C, and 5%, respectively. RPMI-1640 medium containing fetal bovine serum (10%), streptomycin (100 mg/ml), glutamine (2 mmol/l), and penicillin (100 U/ml). was used to cultivate these cells.

Cells were overexpressed with CDKN2B-AS1 and miR-181a by transfecting pcDNA-CDKN2B-AS1 vector or the mimic of miR-181a. Lipofectamine 2000 (Invitrogen, California, USA) was used to perform all transfections, and in each transfection 10^6 cells were incubated with 50 mmol/l miRNA or 20 mmol/l vector.

Table 1 Comparison of the clinical data between polycystic ovary syndrome and control groups

Features	PCOS	Control
Age	27.1 ± 3.4	27.0 ± 3.7
BMI	23.38 ± 4.99	23.12 ± 3.98
Basal E2 (pg/ml)	58.11 ± 17.45*	40.11 ± 13.21
Basal antral follicle count	28.34 ± 6.45*	14.45 ± 3.34
Basal testosterone (nmol/ml)	1.99 ± 0.36*	0.87 ± 0.49
Basal FSH (mlU/ml)	5.749 ± 1.22*	7.23 ± 2.01
Basal LH (mlU/ml)	9.23 ± 5.12*	4.34 ± 2.99

LH, luteinizing hormone; LSH, follicle-stimulating hormone; PCOS, polycystic ovary syndrome.
*$P < 0.05$.

RNA preparations, reverse transcriptions, and quantitative PCRs

EZ Tissue/Cell Total RNA Miniprep Kit was applied for the isolations of total RNA from both tissue samples and cultivated cells. In each RNA extraction, 20 mg tissue or 10^7 cells were used. Chaotropic salts in silica membrane selectively bind RNA. DNA contamination was eliminated by DNase digestion. RNase-free water was used to elute RNA. RNA concentration and integrity were analyzed using 2100 Bioanalyzer System (Agilent, California, USA). RNA concentration was higher than 500 ng/µl in all cases, and RNA integrity value higher than 9.0 was reached.

AzuraFlex cDNA Synthesis Kit (Azura Genomics, USA) was applied to prepare cDNA samples through reverse transcriptions (RTs) using total RNA samples as template. All RTs were performed through following protocol: (a) 0.5 µg total RNA, 1 µl primer, and RNase-free water were used to prepare a 12-µl system, which was incubated for 2 min at 70 °C; (b) 5× RTase Buffer and 1-µl RTase were added to make a 20-µl system, which was incubated for 60 min at 42 °C and then 10 min at 72 °C. Quantitative PCR (qPCR) systems were prepared using 1-µl cDNA in a 20-µl total volume. The expression of CDKN2B-AS1 and miR-181a was analyzed by performing qPCRs with 18S rRNA and U6 as internal controls, respectively. Normalizations of Ct values of targeted genes to corresponding internal controls were performed using 2-delta delta Ct method.

RNA–RNA pull-down assay

The preparation of in-vitro transcripts of both CDKN2B-AS1 and a negative control (NC) RNA was performed using SP6/T7 Transcription Kit (Sigma–Aldrich, St. Louis, USA). A 20-µl in-vitro transcription system was incubated at 37 °C overnight, followed by the purification of RNA using Monarch RNA Cleanup Kit (NEB, USA). RNase-free water was used to elute RNA transcripts. Biotin-labeled CDKN2B-AS1 and NC RNA (Bio-CDKN2B-AS1 and Bio-NC) were prepared using Biotin 3' End DNA Labeling Kit (Life Technologies, Waltham, USA), followed by RNA purification using Monarch RNA Cleanup Kit (NEB). Both RNAs were transfected into cells. Cell lysis was carried out 48-h posttransfection, followed by using probe-coated magnetic beads to pull-down RNA complex, which was used to prepare RNA samples. After that, cDNA samples were prepared, and qPCRs were performed to determine miR-181a expression.

Subcellular fractionation assay

Two fractions of cells, including nuclear and cytoplasm, were prepared with Nuclear & Cytoplasmic Extraction Kit (G-Biosciences, St. Louis, USA). In each reaction, 2×10^6 cells were used. Briefly, cell lysis was performed, followed by centrifugation at 10 min for 1200 g to collect supernatant, which was the cytoplasm sample. Nuclear fraction, which was the cell pellet, was further subjected

to nuclear lysis. After that, cell fractions were used to isolate RNA samples, followed by RTs and PCRs to detect CDKN2B-AS1 expression. Electrophoresis was performed using 2% agarose gel to separate PCR products. After EB staining, images were taken with MyECL imager, Waltham, Massachusetts, USA.

Dual-luciferase activity assay

Luciferase vector of CDKN2B-AS1 was constructed using pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Wisconsin, USA). Cells were cotransfected with CDKN2B-AS1 luciferase vector and miR-181a mimic or NC miRNA. Luciferase activity was detected and normalized at 48-h posttransfection.

BrdU incorporation analysis for cell proliferation

Determination of 5-bromodeoxyuridinc (BrdU) incorporation was performed to analyze cell proliferation. Briefly, cells were collected at 48-h posttransfection, after cell resuspension in fresh medium, followed by cell culture for further 24 h. After that, 10 μmol/l BrdU was added into cell culture medium. Incubation was performed for further 2 h, and then peroxidase-coupled anti-BrdU antibody (Sigma–Aldrich) was added to perform 1-h incubation. After PBS washing for three times, tetramethylbenzidine was added for 30 min incubation. Finally, optical density values were measured to reflect cell proliferation.

Statistical analysis

Student's *t*-test was applied to compare two groups. Comparisons among three or more groups were performed using analysis of variance Tukey's test. $P < 0.05$ was statistically significant.

Results

Analysis of the expression of CDKN2B-AS1 and miR-181a, and their correlations

Follicular fluid samples from both PCOS patients ($n = 60$) and controls ($n = 60$) were subjected to the preparation of RNA samples, followed by RTs and qPCRs to explore the differential expression of CDKN2B-AS1 and miR-181a. Our data analysis revealed the overexpressed CDKN2B-AS1 (Fig. 1a; $P < 0.01$) and under-expressed miR-181a (Fig. 1b; $P < 0.01$) in PCOS samples compared with control samples. Correlations between CDKN2B-AS1 and miR-181a across both PCOS and control samples were analyzed with Pearson's correlation coefficient. Interestingly, they were not closely correlated with each other across PCOS (Fig. 1c) and control (Fig. 1d) samples.

Analysis of the subcellular location of CDKN2B-AS1 in granulosa cells, and its interaction with miR-181a

Subcellular location determines function. Therefore, we first analyzed the subcellular locations (nuclear and cytoplasm) of CDKN2B-AS1 in granulosa cells by performing subcellular fractionation assay. Different form glyceraldehyde-3-phosphate dehydrogenase, which is a cytoplasm marker, CDKN2B-AS1 was detected in both nucleus and cytoplasm, suggesting its function in both locations (Fig. 2a). IntaRNA 2.0 was applied to explore the direct interaction between CDKN2B-AS1 and miR-181a, and a strong direct interaction was predicted (Fig. 2b). RNA–RNA pull-down assay was applied to confirm the direct interaction between them. Interestingly, miR-181a expression level was significantly higher in Bio-CDKN2B-AS1 pull-down group compared to Bio-NC pull-down group (Fig. 2c, $P < 0.01$), which confirmed the direct interaction between them. Dual-luciferase activity assay was performed by cotransfecting cells with CDKN2B-AS1 luciferase vector and miR-181a mimic (miR-181a group) or NC miRNA (NC group). Luciferase activity was detected and normalized at 48-h posttransfection. Compared with NC group, significantly reduced luciferase activity was detected in miR-181a group (Fig. 2d, $P < 0.01$), further confirming the direct interaction between them.

Analysis of the regulatory role of CDKN2B-AS1 and miR-181a in each other's expression in granulosa cells

The direct interaction between CDKN2B-AS1 and miR-181a may indicate their roles in regulating the expression of each other, such as the targeting of CDKN2B-AS1 by miR-181a. To explore this possibility, granulosa cells were overexpressed with CDKN2B-AS1 and miR-181a, and their overexpression was confirmed every 24 h until 96 h (Fig. 3a; $P < 0.05$). In granulosa cells with CDKN2B-AS1 overexpression, no significant alterations in miR-181a expression were noticed at each time points (Fig. 3b). Similarly, cells with miR-181a overexpression also showed no significant alterations in the expression of CDKN2B-AS1 (Fig. 3c).

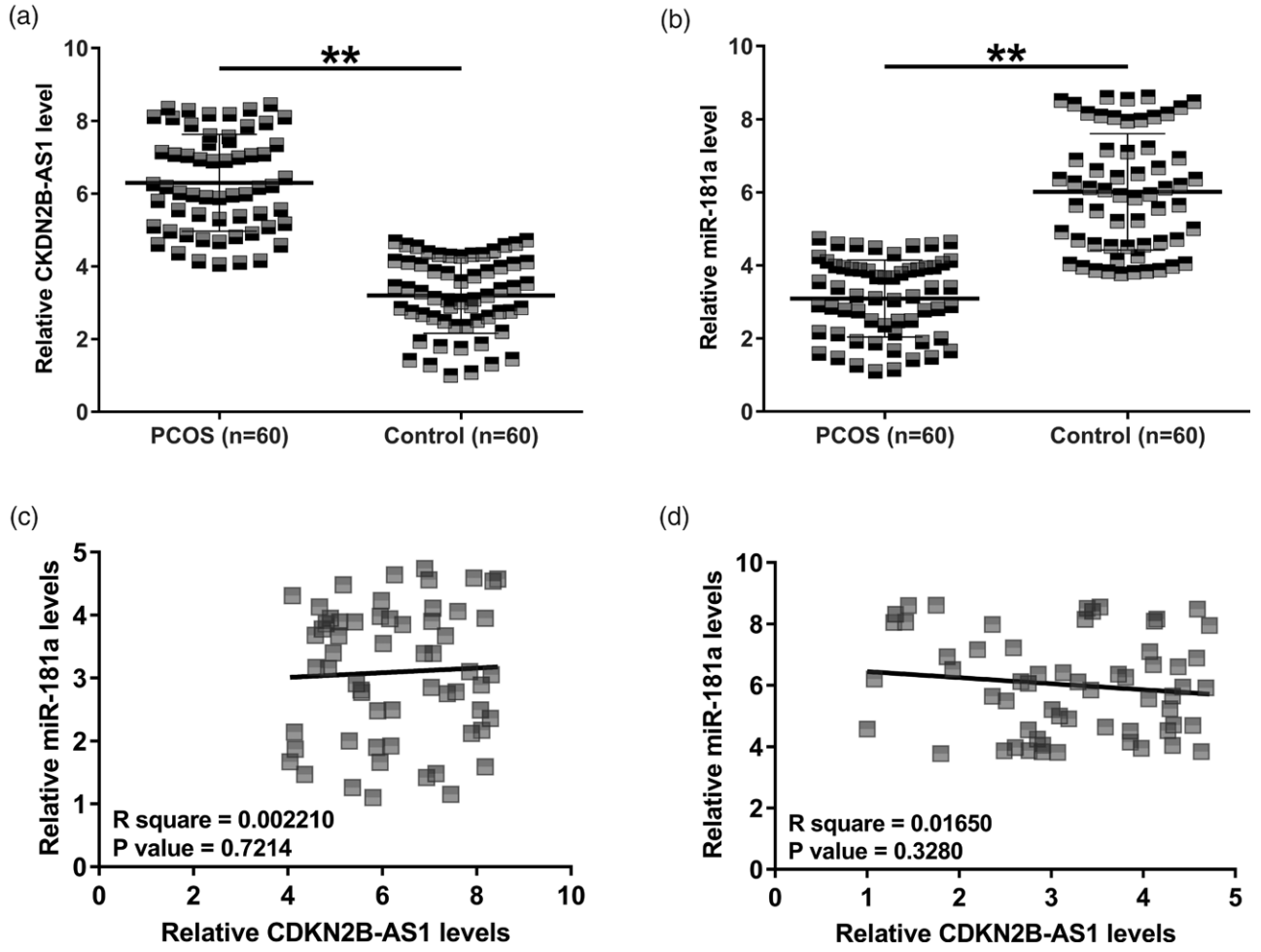
Analysis of the proliferation of granulosa cells with CDKN2B-AS1 and/or miR-181a overexpression

The proliferation of granulosa cells with CDKN2B-AS1 and/or miR-181a overexpression was explored with BrdU assay. Our data analysis revealed the increased proliferation of granulosa cells after CDKN2B-AS1 overexpression and the decreased proliferation of cells after miR-181a overexpression. In addition, the inhibitory effect of miR-181a on granulosa cell proliferation was attenuated by CDKN2B-AS1 (Fig. 4; $P < 0.05$).

Discussion

The present study explored the functionality of both CDKN2B-AS1 and miR-181a in PCOS and analyzed the potential interaction between them. We observed the altered expression of CDKN2B-AS1 and miR-181a in PCOS. In addition, CDKN2B-AS1 may serve as an endogenous competing RNA of miR-181a in granulosa cells to promote cell proliferation, thereby participating in PCOS.

Fig. 1

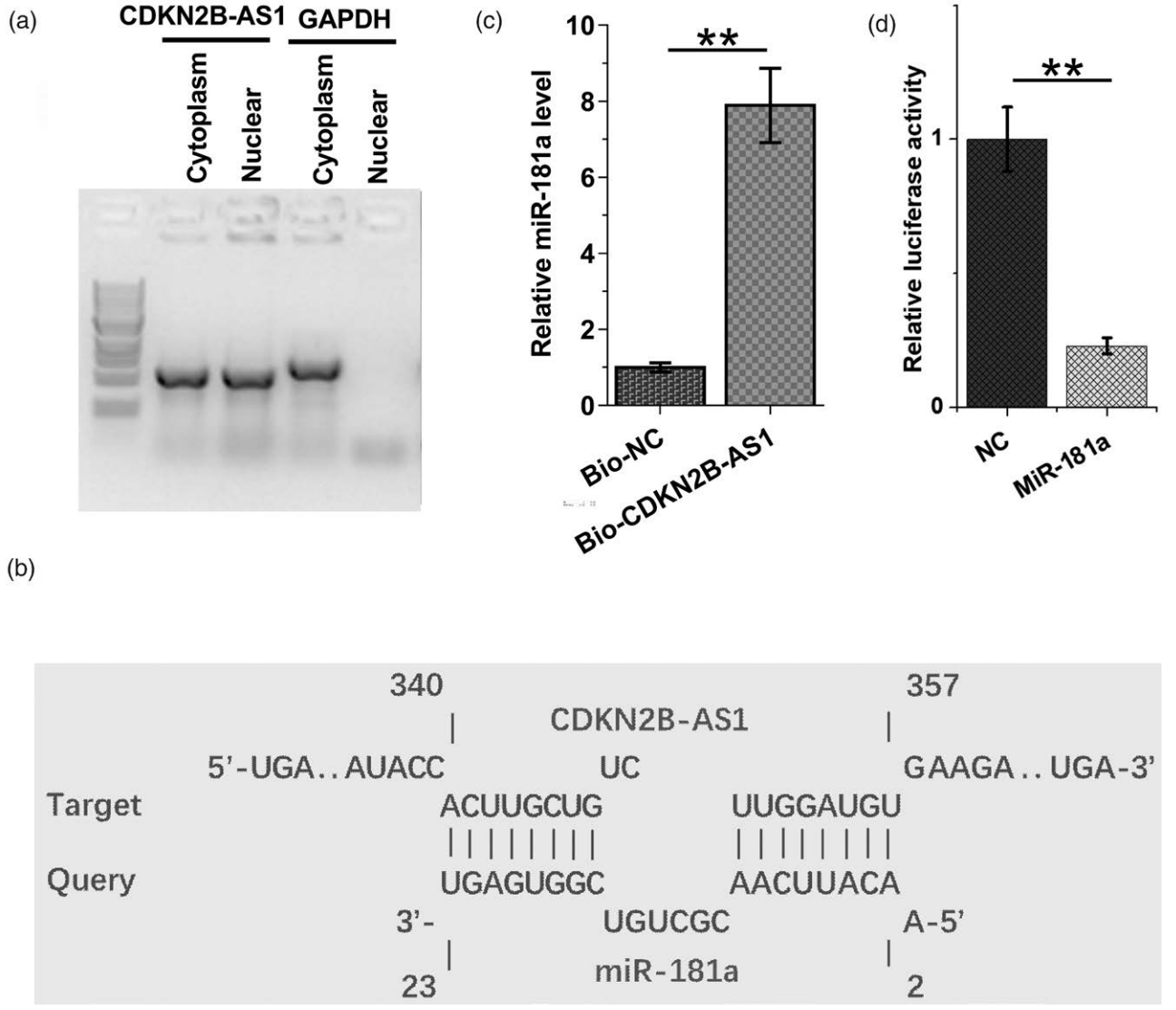


Analysis of the expression of CDKN2B-AS1 and miR-181a, and their correlations. Follicular fluid samples from both PCOS patients ($n = 60$) and controls ($n = 60$) were subjected to the preparation of RNA samples, followed by RTs and qPCRs to explore the differential expression of CDKN2B-AS1 (a) and miR-181a (b). Correlations between CDKN2B-AS1 and miR-181a across both PCOS (c) and control (d) samples were analyzed with Pearson's correlation coefficient. $^{**}P < 0.01$. PCOS, polycystic ovary syndrome; qPCR, quantitative PCR; RT, reverse transcription.

Although no previous studies have reported the involvement of miR-181a in PCOS, the role of miR-181a in the behaviors of granulosa cell has been reported by previous studies [14,16]. MiR-181a targets activin receptor IIA to suppress the proliferation of mouse granulosa cells [14]. In another study, miR-181a was reported to downregulate sphingosine-1-phosphate receptor 1, thereby promoting the proliferation of follicular granulosa cells [16]. Granulosa cells produce LH receptors and steroids to guide the normal development of the oocyte [17]. Altered proliferation and apoptosis promote the progression of PCOS [17]. Therefore, we speculated that miR-181a may participate in PCOS. In this study, we confirmed the inhibitory effects of miR-181a on the proliferation of granulosa cells. We also showed the decreased expression of miR-181a in PCOS. Therefore, altered expression of miR-181a may regulate granulosa cell proliferation to participate in PCOS. PCOS is characterized by the increased proliferation of granulosa cells [1–4]. Therefore, overexpression of miR-181a may sever as a potential target to treat PCOS. However, clinical trials are needed to explore the potential of miR-181a in PCOS treatment.

Although the role of miR-181a in the proliferation of granulosa cells has been reported [14,16], the upstream regulators involved in this process are unknown. CDKN2B-AS1 is a well-characterized lncRNA in cancer biology, including ovarian cancer [18]. In a recent study, CDKN2B-AS1 was reported to upregulate AKT3 by sponging miR-424-5p, thereby promoting epithelial-mesenchymal transition in endometriosis [15]. It has been reported that altered endometrial function also contributes to PCOS [19]. Therefore, CDKN2B-AS1 may also participate in PCOS. The present study showed the

Fig. 2



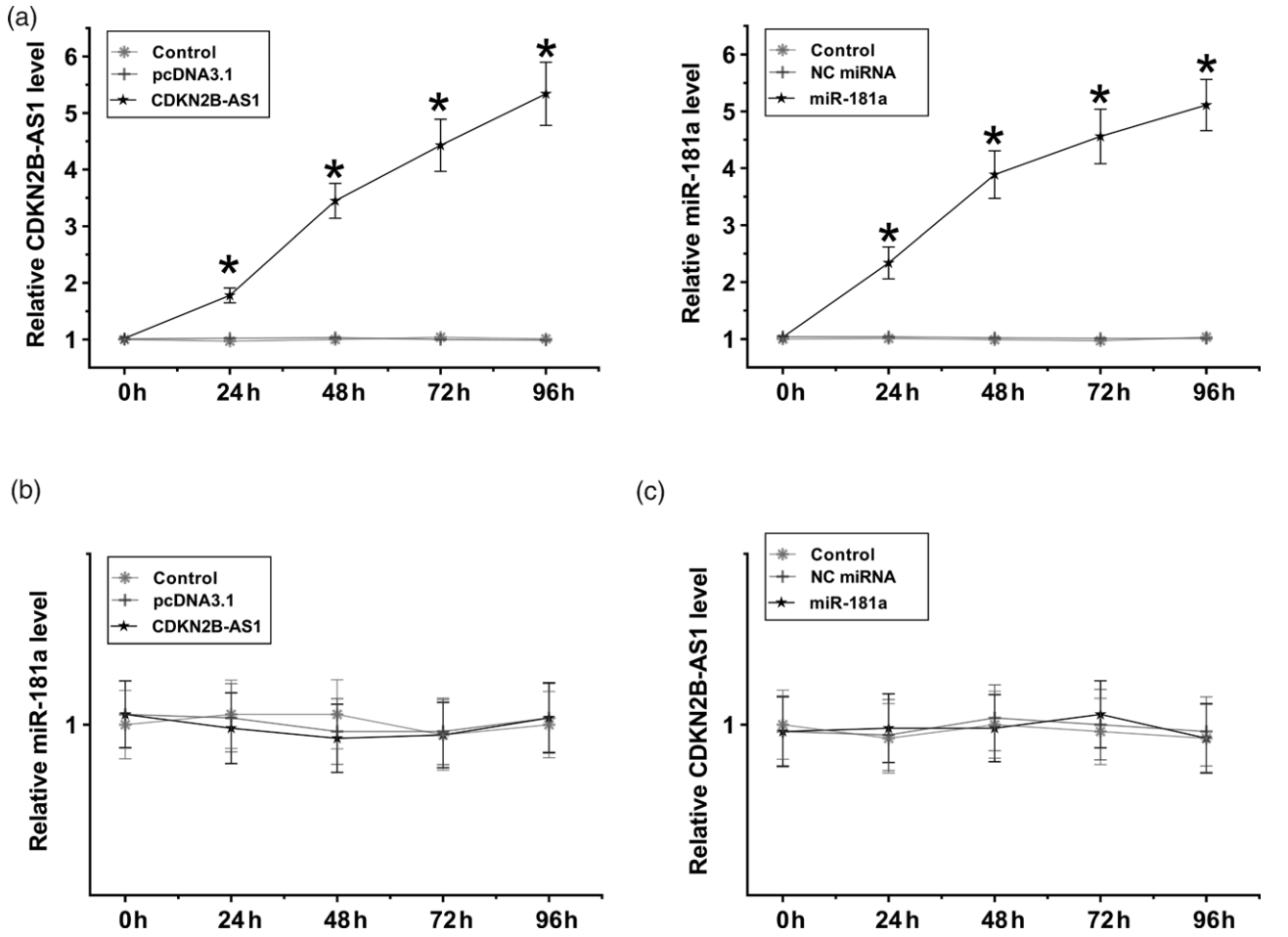
Analysis of the subcellular location of CDKN2B-AS1 in granulosa cells, and its interaction with miR-181a. The subcellular locations (nuclear and cytoplasm) of CDKN2B-AS1 in granulosa cells was analyzed by performing subcellular fractionation assay (a). IntaRNA 2.0 was applied to predict the direct interaction between CDKN2B-AS1 and miR-181a (b), and the direct interaction between them was confirmed by RNA–RNA pull-down assay (c) and dual-luciferase activity assay (d). $^{**}P < 0.01$.

increased expression levels of CDKN2B-AS1 in PCOS and also revealed the enhancing effects of CDKN2B-AS1 on the proliferation of granulosa cells. Therefore, overexpression of CDKN2B-AS1 may serve as a potential target to PCOS by promoting the proliferation of granulosa cells to maintain the normal function of ovary.

Interestingly, we showed that CDKN2B-AS1, which could be detected in both nuclear and cytoplasm, could directly interact with miR-181a in granulosa cells. However, in granulosa cells, they failed to regulate the expression of each other, whereas CDKN2B-AS1 suppressed the role of miR-181a in cell proliferation. It has been well established that the function of miRNA sponges, or endogenous competing RNAs, is to suppress the function of miRNAs by directly absorbing them, but may or may not affect their expression [20,21]. We, therefore, concluded that CDKN2B-AS1 in cytoplasm may sponge miR-181a in cytoplasm to suppress its function.

However, our study only includes a small number of patients. Therefore, future studies with more patients are needed to further confirm our conclusions. In addition, animal model experiments may be performed to further explore the in-vivo interaction bewteen CDKN2B-AS1 and miR-181a in PCOS.

Fig. 3



Analysis of the regulatory role of CDKN2B-AS1 and miR-181a in each other's expression in granulosa cells. Granulosa cells were overexpressed with CDKN2B-AS1 and miR-181a, and their overexpression was confirmed every 24 h until 96h (a). The expression of miR-181a in granulosa cells with CDKN2B-AS1 overexpression (b) and the expression of CDKN2B-AS1 in granulosa cells with miR-181a overexpression (c) were analyzed with RT-qPCR. *$P < 0.05$. RT-qPCT, reverse transcription-quantitative PCR.

In conclusion, CDKN2B-AS1 is highly expressed in PCOS, and miR-181a is lowly expressed in PCOS. CDKN2B-AS1 may sponge miR-181a in granulosa cells to suppress its inhibitory role in cell proliferation, thereby participating PCOS.

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Ethics approval and informed consent: this study passed the review of the Ethics Committee of Suzhou Hospital of Integrated Traditional Chinese and Western Medicine and was carried out following the World Medical Association Declaration of Helsinki. All participants received written informed consent and provided written informed consent.

Availability of data and material: the datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of interest

There are no conflicts of interest.

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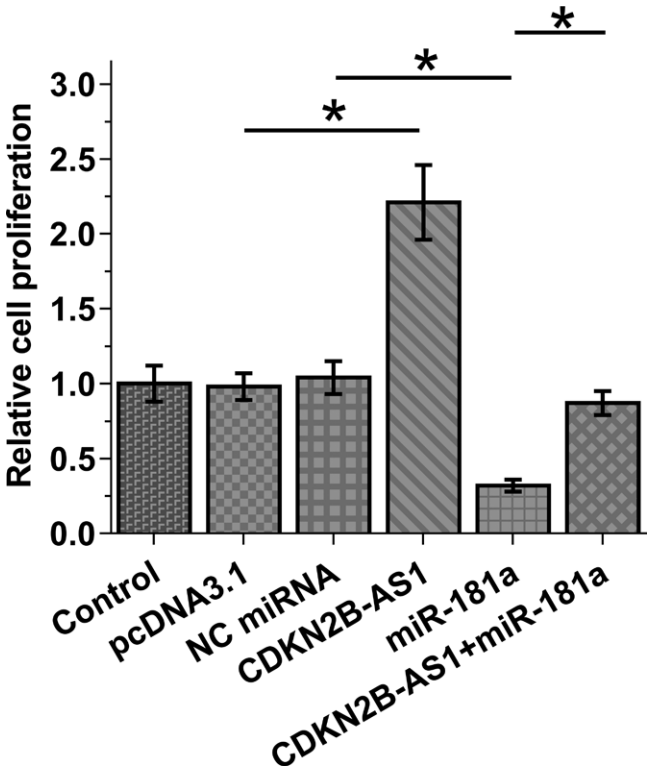
Fig. 4



Analysis of the proliferation of granulosa cells with CDKN2B-AS1 and/or miR-181a overexpression. The proliferation of granulosa cells with CDKN2B-AS1 and/or miR-181a overexpression was explored with BrdU assay. Data of three replicate experiments were presented. *$P < 0.05$. BrdU, 5-bromodeoxyuridinc.

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