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The role of MiR-324-3p in polycystic ovary syndrome (PCOS) via targeting WNT2B

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Abstract. – OBJECTIVE: To investigate the expression of microRNA-324-3p (miR-324-3p) in polycystic ovary syndrome (PCOS) and its effects on the proliferation and apoptosis of ovarian granulosa cells.

MATERIALS AND METHODS: A total of 60 Sprague-Dawley (SD) rats were randomly divided into normal group (n=30) and experimental group (n=30). Rats in the experimental group were intramuscularly injected with dehydroepiandrosterone (DHEA) (6 mg/100 g of body weight) and 0.2 mL oil for injection, while those in normal group were intramuscularly injected with 0.2 mL oil for injection. The ovarian tissues of PCOS model rats were removed to extract the total ribose nucleic acid (RNA). The expression of miR-324-3p was detected via reverse transcription-polymerase chain reaction (RT-PCR). Primary ovarian granulosa cells were isolated and cultured, and NC-miRNA and miR-324-3p mimic were transfected into cells. After 48 h, cell proliferation and apoptosis were detected via cell counting kit 8 (CCK-8) and flow cytometry assay, respectively. The targeted molecule of miR-324-3p was explored using bioinformatics, and dual-luciferase assay was performed to verify the effect of miR-324-3p on WNT2B expression. Granulosa cells were co-transfected with WNT2B-small-interfering RNA (siRNA) and miR-324-3p mimic, and then cell proliferation and apoptosis were detected via CCK-8 and flow cytometry assay, respectively.

RESULTS: The expression of miR-324-3p in ovarian tissues of PCOS group was significantly lower than that of normal group ($p < 0.01$). After transfection with miR-324-3p mimic into granulosa cells, cell proliferation was significantly inhibited and cell apoptosis was promoted ($p < 0.01$). MiR-324-3p exerted its effect on granulosa cells by directly targeting WNT2B. Silencing WNT2B expression could reverse the effects of miR-324-3p on proliferation and apoptosis of granulosa cells ($p < 0.05$).

CONCLUSIONS: The expression of miR-324-3p in the ovary of PCOS rats is decreased significantly. Overexpression of miR-324-3p can reduce the proliferation and induce the apoptosis of granulosa cells via targeting of WNT2B.

Key Words:
GMiR-324-3p, PCOS, Proliferation, Apoptosis, WNT2B.

Introduction

Polycystic ovary syndrome (PCOS) is a kind of common endocrine disease in women of childbearing age. Its occurrence accounts for about 5-10% of diseases in women of childbearing age[1,2]. Currently, there are three different criteria for the diagnosis of PCOS, namely National Institutes of Health (NIH) criteria[3], Rotterdam criteria proposed by the European Society of Human Reproduction and Embryology/American Society for Reproductive Medicine (ESHRE/ASRM)[4] and Androgen Excess Society (AES) criteria[5]. Hyperandrogenism, ovulation failure and polycystic ovary morphological changes are three bases of clinical diagnosis meeting criteria. PCOS women are often accompanied with insulin resistance, hyperinsulinemia, obesity and other characteristics, all of which significantly increase the risks of cardiovascular disease and type 2 diabetes mellitus in patients[6]. The pathogenesis of PCOS is more complex, and it has not been very clear so far. However, it is widely recognized that environmental factors and genetic factors play important roles in the occurrence of PCOS. Therefore, in recent years, the specific mechanism causing PCOS has become a research hotspot.

Normal reproductive function is a multi-factor and complex process. The accurate expressions of sex hormones, cytokines and genes are necessary for the development and function of normal reproductive organs and the development of follicles and embryos. Disorders of these factors can lead to reproductive dysfunction, resulting in

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infertility, PCOS, endometriosis and ovarian tumor. Some reports[7-10] have found that many genes are associated with the pathogenesis of PCOS. By using gene chips it has been found that there are differential expressions of more than a hundred genes in women with PCOS compared with normal people. These genes are involved in a variety of biological processes, such as cell division, apoptosis and metabolism[7-10].

New evidence[11,12] has shown that micro ribonucleic acids (miRNAs) from different mammalian species in the ovary play important roles in post-transcriptional gene regulation of ovarian disease, such as PCOS. Previous investigations[13] have shown that the miR-324-3p level in serum of patients with PCOS declines, proving that it may be closely related to PCOS. In this research, the expression of miR-324-3p in PCOS and its effects on the proliferation and apoptosis of ovarian granulosa cells were investigated and its mechanism was further explored, providing a theoretical basis for the treatment of PCOS.

Materials and Methods

Main Reagents

Fetal bovine serum (FBS) and Dulbecco's modified Eagle medium (DMEM)/F12 medium were purchased from Gibco (Rockville, MD, USA). Cell counting kit-8 (CCK-8) cell proliferation kits, bicinchoninic acid (BCA) kits and Annexin V-fluorescein isothiocyanate (FITC) kits were purchased from Beyotime Institute of Biotechnology (Shanghai, China). WNT2B and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Abcam (Cambridge, MA, USA). Fluorescence quantitative kits and reverse transcription kits were purchased from Bio-TNT (Shanghai, China).

Establishment of PCOS Model and Specimen Collection

A total of 60 clean-grade Sprague-Dawley (SD) female rats weighing 200-250 g were purchased from Shanghai Laboratory Animal Center (SLAC, Shanghai, China). After SD rats aged 21 days old were fed adaptively for 2 d, they were randomly divided into normal group (n=30) and experimental group (n=30). Rats in experimental group were intramuscularly injected with dehydroepiandrosterone (DHEA) (6 mg/100 g of body weight) and 0.2 mL oil for injection, while those in normal group were intramuscularly injected with 0.2 mL oil for injection. After 21 d, rats were fasted for 12 h and the ovaries of rats were removed for standby application. This study was approved by the Animal Ethics Committee of The Children and Women's Health Care Hospital of Laiwu City Animal Center (Laiwu, China).

MiR-324-3p Expression in Ovarian Tissues

The total RNA was extracted from ovarian tissues using the RNA extraction kit (Thermo Fisher Scientific, Waltham, MA, USA), and the concentration of RNA was detected using an ultraviolet spectrophotometer. The optical density $(OD)_{260\ nm}/OD_{280\ nm}$ between 1.8 and 2.0 indicated good-quality samples. The total ribose nucleic acid (RNA) was reversely transcribed into complementary deoxyribonucleic acid (cDNA) according to instructions of the reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). The primer and internal reference primer U6 of miR-324-3p were designed by Bio-TNT (Shanghai, China). The cycle threshold (Ct) value of the sample was detected using the fluorescence quantitative polymerase chain reaction (PCR) instrument, and relatively quantitative analysis was performed for the mean value using $2^{-\Delta\Delta Ct}$ method. $2^{-\Delta\Delta Ct}$ = relative expression of gene, $^{\Delta\Delta Ct} = (Ct_{target\ gene} - Ct_{internal reference\ gene})$ experimental group - $(Ct_{target\ gene} - Ct_{internal reference\ gene})$ control group.

Primary Culture of Ovarian Granulosa Cells

After SD female rats were fed adaptively for 2 d, 40 IU pregnant mare serum gonadotropin (PMSG) was injected subcutaneously. After 48 h, rats were executed *via* cervical dislocation, and ovarian tissues were removed and quickly placed into normal saline. The ovary was placed on a super-clean bench, the ovary capsule on the surface and the surrounding adipose tissues were removed under a microscope, and the red blood cells on the surface were washed away with normal saline again. Then, the ovary was placed into the serum-free DMEM (Dulbecco's Modified Eagle Medium)/F12 medium, and the follicle was punctured using a 1 mL injection needle to release follicular granulosa cells. After that,

cells were gently blown and beat using a Pasteur pipette to be prepared into single cell suspension, filtered *via* a 200-mesh sieve, and centrifuged at 1000 rpm for 8 min. The supernatant was discarded and cells were collected. Granulosa cells precipitated were added with DMEM/F12 medium containing 15% fetal bovine serum (FBS) and incubated with 5% CO_2 at 37°C. After 24 h, cells adhered to the wall, adherent cells were removed and remaining cells continued to be cultured. When the cell growth density reached 80%, cells were digested, centrifuged, and collected. Granulosa cells precipitated were added with DMEM/F12 medium, blown and beat using the Pasteur pipette to be prepared into cell suspension, followed by trypan blue staining, microscopic counting, and passage.

Granulosa Cell Transfection

Normal control (NC)-small-interfering RNA (siRNA), WNT2B-siRNA, miR-324-3p-mimic and miR--NC were purchased from Shanghai Genechem Pharmaceutical Co., Ltd (Shanghai, China). Transfection was performed according to the instructions of Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). Cells were collected after 48 h for subsequent detection.

Detection of Cell Proliferation via CCK-8

Cells were inoculated into a 96-well plate. After transfection for 48 h, 10 μL CCK-8 reagent were added into cells in each well for incubation at 37°C for 4 h. Before the determination of OD value in each well, the zero was set using the blank control well, after which the OD value of each group was detected and recorded at a wavelength of 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). The experiment was repeated for 3 times, and the cell proliferation rate was calculated. Cell proliferation rate = $(OD_{\text{experimental group}}/OD_{\text{blank control group}}) \times 100\%$.

Detection of Apoptosis via Flow Cytometry

Cells were inoculated into a 6-well plate. After transfection for 48 h, cells were collected and prepared into cell suspension (3 tubes in each group, 1 mL/tube). 500 μL binding buffer were added to re-suspend cells, and 5 μL propidium iodide (PI) and 5 μL Annexin V-Fluorescein isothiocyanate (FITC) were added for reaction in a dark place at room temperature for 15 min. Finally, cell apoptosis was detected using a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) within 1 h.

Western Blotting

The total protein was extracted from cells using the protein extraction kit (KeyGen Biotech Co., Ltd., Nanjing, China), and the BCA kit was used to detect the concentration of protein extracted. The protein sample and loading buffer were mixed evenly, followed by denaturation at 100°C for 5 min. After separation via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), membrane transfer and sealing, the denatured protein was incubated using primary antibody at 4°C overnight. Then, the secondary antibody was added for incubation at 37°C for 1 h. After the membrane was washed with Tris-buffered saline and Tween-20 (TBST), the color was developed using enhanced chemiluminescence (ECL) agent, followed by development and fixation. Finally, the expression level of target protein was analyzed with GAPDH as an internal reference.

Detection of Effect of miR-324-3p on WNT2B Expression Via Dual-Luciferase Assay

To demonstrate that miR-324-3p specifically acts on the WNT2B 3'-untranslated region (UTR), the dual-luciferase reporter gene system of 3'-UTR (Psi-check2/WNT2B) containing WNT2B mRNA and its 3'-UTR mutant (Psi-check2/mut) was constructed. MiR-324-3p-mimic and Psi-check2/WNT2B or Psi-check2/mut were co-transfected into 293T cells, and the firefly and Renilla fluorescence signals were read using the dual-luciferase assay kit after 24 h. The relative fluorescence value of Renilla/firefly was used for comparison.

Statistical Analysis

All experimental data were analyzed using Statistical Product and Service Solutions (SPSS) 21.0 software (IBM, Armonk, NY, USA). Measurement data were presented as mean ± standard deviation. Comparison between groups was done using One-way ANOVA test followed by Least Significant Difference (LSD), and *t*-test was used for the comparison between two groups. $p < 0.05$ suggested that the difference was statistically significant.

Results

Expression of miR-324-3p in the Ovary Tissues of PCOS Rats

The ovarian tissues of PCOS model rats were extracted, and the total RNA was extracted to detect the expression of miR-324-3p via RT-PCR. Results showed that the expression of miR-324-3p in ovarian tissues was significantly lower than that in normal group ($p < 0.01$) (Figure 1).

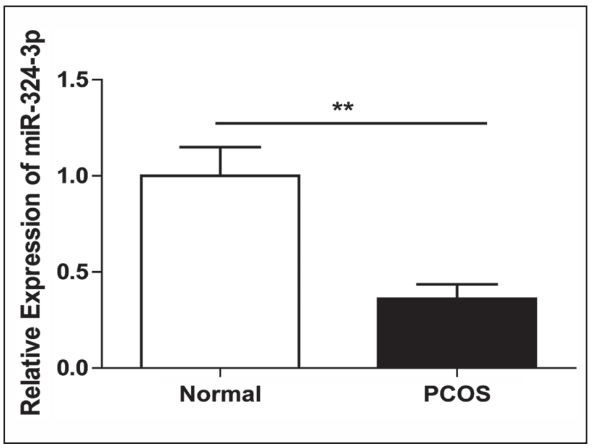

Figure 1. Expression of miR-324-3p in ovary tissues of PCOS rats. RT-qPCR showed miR-324-3p expression was significantly lower in ovary tissues of PCOS rats than in the normal group. $^{**}p < 0.01$.

Effects of miR-324-3p on Proliferation and Apoptosis of Granulosa Cells

Granulosa cells were infected with miR-324-3p mimic, and the expression of miR-324-3p in cells in each group after transfection was detected via RT-qPCR. Results showed that the expression of miR-324-3p in cells transfected with miR-324-3p mimic was significantly increased ($p < 0.05$) (Figure 2A). Results of cell proliferation assay showed that the survival rate of cells transfected

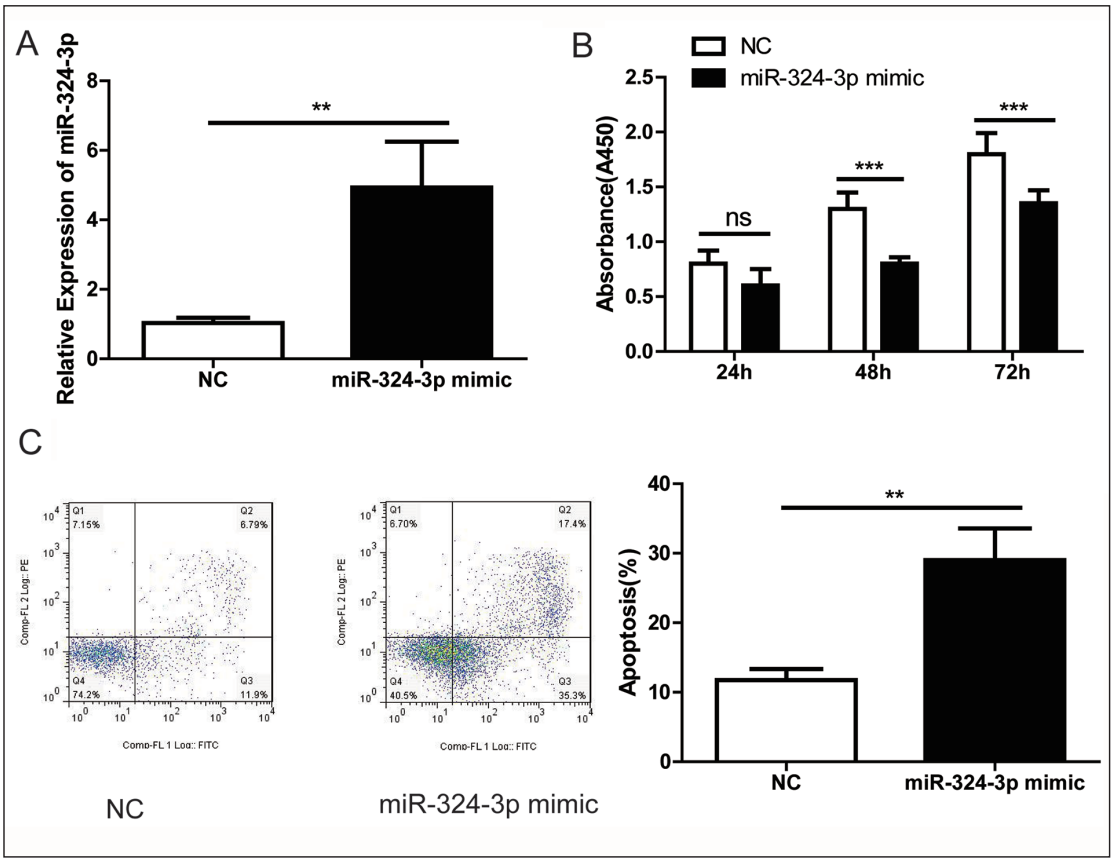

Figure 2. Ectopic expression of miR-324-3p inhibited cell proliferation but increased cell apoptosis of granulosa cells. ***A,*** The relative expression levels of miR-324-3p in granulosa cells were determined by RT-qPCR after transfection with miR-324-3p mimic or miR-NC. ***B,*** The proliferative cell potential *via* CCK-8 assay was determined in granulosa cells transfected with miR-324-3p mimic or miR-NC. ***C,*** The apoptotic rate *via* flow cytometer assay was determined in granulosa cells transfected with miR-324-3p mimic or miR-NC. $^{**}p < 0.01$, $^{***}p < 0.001$.

with miR-324-3p mimic was significantly decreased ($p < 0.001$) 48 and 72 h after transfection (Figure 2B). Besides, apoptosis in each group was detected using the flow cytometer assay, and results showed that the apoptotic rate of cells transfected with miR-324-3p mimic was significantly increased ($p < 0.01$) (Figure 2C).

WNT2B Was a Downstream Regulation Target of miR-324-3p

Bioinformatics detection (Figure 3A) showed WNT2B as a possible target of miR-324-3p. The expression level of WNT2B after transfection with miR-324-3p mimic was detected by Western blotting, and results showed that the expression level of WNT2B was significantly decreased after transfection with miR-324-3p mimic (Figure 3B). Dual-luciferase assay showed that after co-transfection with Psi-check2/WNT2B and miR-324-3p mimic, the Renilla relative fluorescence signal was decreased significantly ($p < 0.01$). The Renilla relative fluorescence signal basically had no change after co-transfection with Psi-check2/mut and miR-324-3p mimic ($p > 0.05$) (Figure 3C). As a result, it is suggested that miR-324-3p may affect the proliferation and apoptosis of granulosa cells through the expression of WNT2B.

Silencing WNT2B Expression Reversed the Effects of miR-324-3p on Proliferation and Apoptosis of Granulosa Cells

Results of CCK-8 assay (Figure 4A) revealed that the proliferation rate of granulosa cells in WNT2B-siRNA + miR-324-3p mimic group was higher than that of granulosa cells in miR-324-3p mimic group, and there were statistically significant differences at 24, 48 and 72 h ($p < 0.001$). At the same time, the apoptosis rate of granulosa cells in WNT2B-siRNA + miR-324-3p mimic group was lower than that of granulosa cells in miR-324-3p mimic group (Figure 3B, $p < 0.05$). The above results suggest that silencing WNT2B expression can reverse the

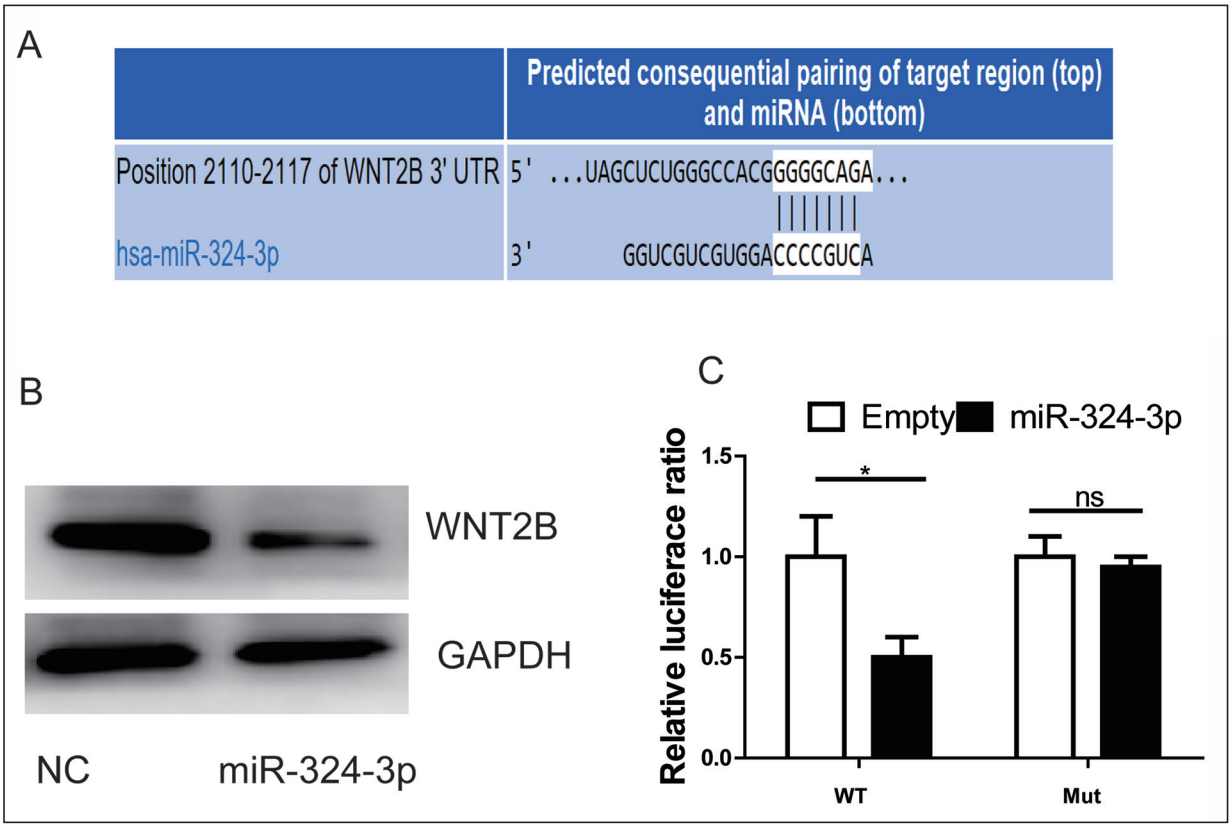

Figure 3. MiR-324-3p downregulates WNT2B expression through targeting its 3'UTR. ***A,*** WNT2B was predicted to be a target gene of miR-324-3p by TargetScan. ***B,*** Protein expression levels of WNT2B in granulosa cells with miR-324-3p transfection. ***C,*** Luciferase activity of wild-type (UTR-WT) or mutant (UTR-mut) WNT2B 3′-UTR reporter gene in 293T cells infected with miR-324-3p. Ns, none significant, *$p < 0.05$.

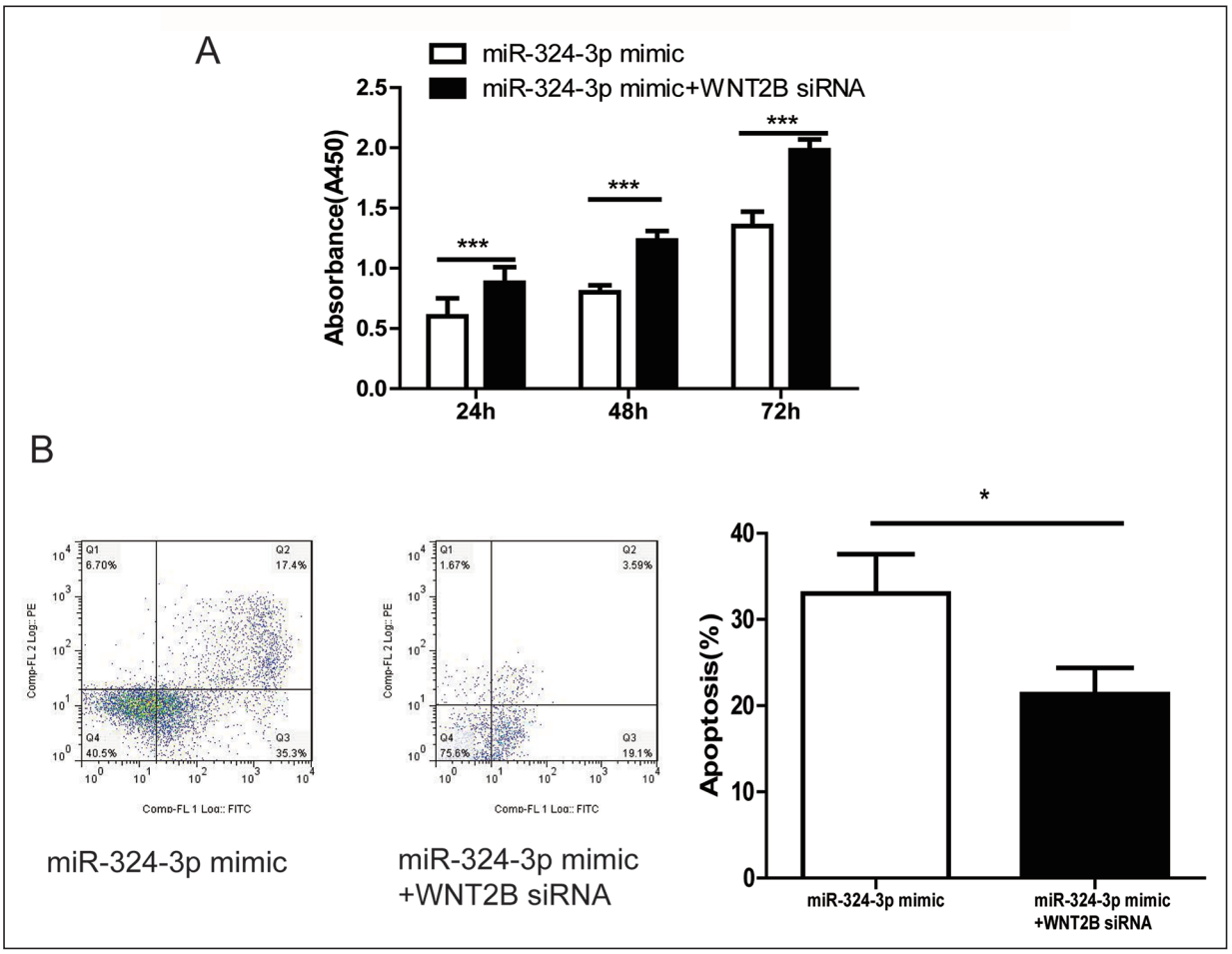

Figure 4. Silencing WNT2B expression reverses the effects of miR-324-3p on proliferation and apoptosis of granulosa cells. ***A,*** The proliferative cell potential via CCK-8 assay was determined in granulosa cells transfected with miR-324-3p mimic with/without WNT2B-siRNA. ***B,*** The apoptotic rate via flow cytometer assay was determined in granulosa cells transfected with miR-324-3p mimic with/without WNT2B-siRNA. *$p < 0.05$, ***$p < 0.001$.

effects of miR-324-3p on proliferation and apoptosis of granulosa cells, and indirectly reflect the regulatory relationship between miR-324-3p and WNT2B.

Discussion

PCOS is the most common reproductive endocrine disease in women of childbearing age, which is a syndrome involving multiple factors and a series of symptoms and signs. Its clinical manifestations and biochemical features are highly heterogeneous, and it has always been a research hotspot in the field of reproductive endocrinology in recent years[14]. Some researchers have found that disorders in the regulatory system of multiple factors in the ovary may be involved in the occurrence of PCOS. According to previous reports, the serum miR-324-3p level in patients with PCOS is decreased, proving that it may be closely related to PCOS[13]. On this basis, it was found in this study that the expression of miR-324-3p in ovarian tissues of PCOS model rats was decreased. In addition, *in-vitro* experiments further clarified that miR-324-3p might regulate the proliferation and apoptosis of granulosa cells *via* targeting WNT2B, thus playing an important role in PCOS.

MiRNA is a class of non-coding single-stranded RNA molecule with a length of 17-25 nucleotides encoded by endogenous genes, which inhibits the translation process of target gene through binding to the 3'-UTR of target gene mRNA, thus regulating target genes and participating in various biological behaviors of the tumor[15]. There

is not a simple one-to-one relationship between miRNA and target gene, because one miRNA can regulate multiple target genes, and one target gene can be regulated by multiple miRNAs. Previous reports[12,16,17] indicated that miRNA may be involved in the pathogenesis of PCOS. In a previous investigation, the PCOS rat model showed that miR-222 was expressed in the follicular membrane and granulosa cells of early follicles, and its expression disappeared in granulosa cells when the follicle developed into mature stage[12].

At present, there has been little research on the function and mechanism of miR-324-3p. In the analysis on non-neoplastic diseases, Cui et al[18] reported that the expression of miR-324-3p combined with another five miRNAs (miR-148a, miR-143, miR-628-3p, miR-140-5p and miR-362-3p) in serum can help distinguish hand-foot-mouth infections caused by enterovirus 71 and Coxsackie virus 16. Researches[19] on renal fibrosis showed that miR-324-3p can reduce Prep to promote the occurrence of renal fibrosis in progressive nephropathy. In tumor research, Hu et al[20] found that there were significant differential expressions in miR-324-3p compared with another three miRNAs (miR-16, miR-25 and miR-222) in serum of patients with breast cancer. Sun et al[21] found that miR-324-3p promotes the progression of gastric cancer through the regulation of activating Smad4-mediated Wnt/beta-catenin signaling pathway. In hepatocellular carcinoma[22], miR-324-3p can also promote tumor cell proliferation by targeting DACT1.

In this study, the expression level of miR-324-3p in ovarian tissues of PCOS model rats was decreased. In *in-vitro* experiments, miR-324-3p mimic could inhibit granulosa cell proliferation, which may be related to follicular development and maturation disorders in PCOS patients. Lin et al[23] detected the difference in miRNA expression profile between normal follicle and atretic follicle in pigs using miRNA microarray deep sequencing. They found that expressions of let-7a, let-7i and miR-92b in atretic follicles were reduced, suggesting that these miRNAs may be involved in inhibiting apoptosis. In addition, some previous reports[17,23] indicated that the apoptosis level of ovarian granulosa cells in PCOS patients was significantly increased. In this study, it was found that miR-324-3p mimic could promote apoptosis of granulosa cells, indicating that miR-324-3p may participate in the regulation of apoptosis, even follicular atresia process and follicular maturation disorder.

We showed that enhancing the miR-324-3p expression could inhibit proliferation and promote apoptosis of granulosa cells. As mentioned above, miR-324-3p can exert its function through regulating the expressions of various cell-signaling molecules. This study showed that WNT2B protein is a direct target gene of miR-324-3p. WNT2B, a member of the WNT family, is the 13th human WNT gene cloned in 19 members, and it plays a very important role in drosophila embryonic development. It has been reported that WNT2B is involved in the regulation of proliferation, apoptosis, metastasis and invasion of various kinds of cells[24,25].

Conclusions

We demonstrated that miR-324-3p may affect the proliferation and apoptosis of granulosa cells by regulating the expression of WNT2B protein, thus participating in the occurrence and development of PCOS. Of course, all of the above studies were *in-vitro* experiments, so whether miR-324-3p has the same function *in vivo* will be a research direction in the future.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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