Silencing circ_0058063 enhances proliferation and inhibits apoptosis in PCOS ovarian granulosa cells

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Research Article

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Abstract

Background Polycystic ovary syndrome (PCOS) is the most common endocrine disease in reproductive-aged women. This study was designed to explore the role of circ_0058063 in PCOS.

Methods We recruited nine PCOS patients and nine no-PCOS patients. The concentrations of follicle stimulating hormone (FSH), testosterone (T), luteinizing hormone (LH), progesterone (P4) and estradiol (E2) were measured by radioimmunoassay. The level of aromatase was detected using an ELISA kit. The proliferation and apoptosis of ovarian granulosa cells were assessed using CCK-8 assay and flow cytometry, respectively. Gene and protein expression were evaluated through RT-qPCR and Western blotting assay.

Results The circ_0058063 level in ovarian granulosa cells and follicular fluid is significantly higher in the PCOS group than the no-PCOS group. Besides, silencing circ_0058063 increases the levels of Aromatase mRNA, P4 and E2 in PCOS ovarian granulosa cells. Additionally, silencing circ_0058063 can promote the proliferation of ovarian granulosa cells in patients with PCOS. Furthermore, silencing circ_0058063 can suppress apoptosis of PCOS ovarian granulosa cells.

Conclusions Silencing circ_0058063 enhances proliferation and inhibits apoptosis in PCOS ovarian granulosa cells. Such findings may offer vital insights into a therapeutic target for PCOS.

1 Background

Polycystic ovary syndrome (PCOS) is the most common endocrine disease in reproductive-aged women (1, 2), affecting approximately 10% of women of childbearing age (3). The most prominent characteristics of PCOS are abnormal follicular development and hyperandrogenism (4, 5). Women with PCOS have short-term and long-term complications of multiple systems throughout the body, including reproduction (hairy, amenorrhea, anovulation, infertility and pregnancy complications), metabolism (type 2 diabetes, cardiovascular disease, and abnormal lipid metabolism) and mental (negative, anxiety), which have brought a heavy burden on individuals and the whole society (4). Presently, PCOS cannot be completely cured. Therefore, the deep exploration of the PCOS development mechanism is particularly significant for the treatment of the disease.

Circular RNAs (circRNAs) constitute a kind of non-coding RNAs, which is named for its closed loop structure, lack of 5’cap ends, and 3’poly A tails (6, 7). Recently, several studies suggest that circRNAs are widely involved in the PCOS progression (8, 9). Research evidence shows that the expression of hsa_circ_0043533 and hsa_circ_0043532 in the PCOS group increased evidently than that in the non-PCOS group, while the non-PCOS group had a remarkably lower hsa_circ_0097636 expression as compared to the PCOS group (8). Research evidence shows that circ_0058063 functions as an oncogene in bladder cancer (10). However, the involvement of circ_0058063 in PCOS is still not well discovered. Our aim is to explore the function of circ_0058063 in patients with PCOS, which may shed new light on the development of PCOS treatment.

2 Methods

2.1 Study Subjects

We recruited nine PCOS patients and nine patients with no-PCOS under the age of 35 who underwent in vitro fertilization and embryo transfer (IVF-ET) in the Huai'an Second People's Hospital between January 2018 and April 2019. We measured the subjects' weight, height, and then calculated body mass index (BMI). Selection criteria for the PCOS group (refer to Rotterdam criteria (11)):1) rare ovulation and/or anovulation; 2) clinical manifestations of hyperandrogenism and/or biochemical changes; 3) polycystic ovary was found by ultrasound examination. Those who met 2 of the above 3 items were enrolled in the PCOS group. The no-PCOS group was subjects with normal ovaries function, no PCOS, no history of ovarian adverse reactions, no endometriosis, and receiving IVF-ET due to male factors. All patients voluntarily participated in this research project and signed informed consent. This study was approved by the Ethics Committee of the Huai'an Second People's Hospital.

2.2 Collection of serum

5 mL of fasting venous blood was collected on the morning of the third day of the menstrual cycle. Blood samples were centrifuged at 1500 r/min for 5 min to draw the upper layer of serum, aliquoted into a 0.5 mL EP tube, and stored in the refrigerator at -80 °C for testing.

2.3 Acquisition of follicular fluid

All subjects induced ovulation according to the GnRH-a/FSH/ HMG/HCG protocol. The dosage and time were adjusted based on the individual situation. 36 hours after HCG injection, the follicles of both ovaries were sucked through the posterior vaginal foramen under the guidance of ultrasound. The first tube of clear follicular fluid without blood staining was collected and centrifuged at 400x g for 10 min at room temperature. The supernatant was stored at -80 °C until detecting.

2.4 Collection, separation and purification of ovarian granulosa cells

We collected the discarded follicle puncture fluid after the separation of the ovarian crown complex, centrifuged it at 2000 rpm for 10 min, and then discarded the supernatant. The cells were resuspended in PBS and digested using 0.25% trypsin solution at 37℃ for 5 ~ 10min. After termination of digestion, the cell suspension was centrifuged at 2000 rpm for 10min and resuspended in PBS. Next, we added an equal volume of human lymphocyte separation solution (Tianjin Med Pacific Technology, China) to cells. After centrifugation at 2000 rpm for 20 min, the intermediate cell layer was drawn. The cells were washed with DMEM (Gibco, USA) containing 1% Pen-Strep (Invitrogen, USA) and 10% FBS, centrifuged at 1000 rpm for 10 min, and resuspended with DMEM. Trypan blue staining proved that the cell survival rate was greater than 90%. The cell density was adjusted to 2×10^5 / mL and fostered in a 37 °C, 5% CO_2 incubator. After the cells adhered to the growth, they were rinsed once or twice every 24h and then replaced with fresh culture medium to further purify the mixed blood cells and other impurities.

2.5 Cell transfection

Ovarian granulosa cells in the PCOS group were randomly divided into three groups: si-negative control (NC) group (transfected with NC-siRNA), si-circ_0058063-1 group (transfected with circ_0058063-1-siRNA), si-circ_0058063-2 group (transfected with circ_0058063-2-siRNA). Cells were transfected by Lipofectamine^{TM} 2000 (Invitrogen, USA) reagent according to the manufacturer's instructions.

2.6 Hormone levels detection

The concentrations of follicle stimulating hormone (FSH), testosterone (T), luteinizing hormone (LH), progesterone (P4) and estradiol (E2) were measured by radioimmunoassay. The concentration of aromatase was detected using an ELISA kit. The kits were provided by Xiamen Huijia Biological Technology (Xiamen, China).

2.7 RT-qPCR

After 24-hour transfection, the culture medium was replaced, and cells were continued to foster for 48 hours. The total RNA was extracted by Trizol (Invitrogen, USA), and the RNA quality was detected and quantified by ultraviolet spectrophotometry. The cDNA was synthesized using PrimeScript™ RT Master Mix (Takara, China), and qPCR was performed utilizing TB Green® Premix Ex Taq™ (Takara, China) according to the standard protocol. U6 and β-actin were used as internal reference controls. The relative expression of circ_0058063 and Aromatase mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.8 Western blotting

The total protein of the cell was extracted using RIPA buffer (Biyuntian, China). The protein concentration was assessed via a BCA kit (Biyuntian, China). Next, 50μl protein samples were taken for SDS-polyacrylamide gel electrophoresis to separate the target protein, and the membrane was transferred by the wet transfer method. We sealed the membrane with 5% skimmed milk powder for 2 hours, washed the membrane 3 times with PBST solution, and added primary antibodies (1:1000, CST, USA), including Cyclin A, Cyclin D1, Bcl-2, Bax, and GAPDH. Primary antibodies were incubated at 4 ℃ overnight. Then a secondary antibody (1: 3000) was added and incubated at 37 °C for 2 hours. The ECL kit obtained from Amersham Biosciences (Piscataway, NJ) was applied to visualize the immunocomplexes.

2.9 Colony formation assay

Cells were seeded at colony forming density (200 cells/ dish) in a 60-mm tissue-culture dish carrying 5 mL medium at 37 °C under 5% CO_2. After 14 days, the colony formation rate was observed in each group. Cells were fixed with 4% paraformaldehyde for 15 min after discarding the medium. Next, cells were stained with 0.1% crystal violet for 20 min. In each well, five randomly fields were selected to calculate the number of colonies. Colony formation rate = (number of colonies/number of inoculated cells) × 100% (12).

2.10 Cell-Counting Kit-8 Assay

The cell viability was detected using CCK-8 (Biyuntian, China) based on the instructions. In short, after transfection for 48 hours, 10 μl CCK8 reagent was added to ovarian granulosa cells in each well. The cells were incubated at 37 °C for 4 hours. The microplate reader (Bio-Rad, USA) was used to detect the optical density (OD) at a wavelength of 450 nm.

2.11 Flow cytometry

Cell apoptosis was measured utilizing the Annexin V-FITC Apoptosis Detection Kit (Abcam, USA) as the manufacturer's protocol. In short, we added pre-chilled 1xbinding buffer to resuspend the cells, and then added 10μl PI and 5μl Annexin V-FIT. Cells were stained for 15 ~ 25min in the darkroom. Flow cytometry (BD, USA) was used to detect the apoptosis rate.

2.12 The establishment of PCOS mouse model

Fifty 25-day immature female BALB/c mice were purchased from Wuhan University Center for Animal Experiment. Mice were randomly divided into PCOS group (n=40) and control group (n=10). Mice in the PCOS group were injected with 6 mg DHEA per 100 g body weight (purchased from Hangzhou Aladdin Information Technology Co., Ltd., China) every day for 20 consecutive days to establish a PCOS mouse model. The control group was injected subcutaneously with 0.4 mL of normal saline daily for 20 days. The fasting blood glucose and fasting insulin levels were measured 3 weeks after the injection. HOMA-IR = FI (mU/L) × FG (mmol/L) / 22.5 (13). Insulin resistance is defined as HOMA-IR> 2.6 (14). Thirty PCOS mice with HOMA-IR> 2.6 were randomly divided into si-NC group, si-circ_0058063-1 group and si-circ_0058063-2 group, with 10 mice in each group. Then 5×10^8 PFU/mL lentivirus containing si-circ_0058063 (GenePharma, China) or negative control vector was injected to the mice ovary. Mice were detected IR and then sacrificed 2 weeks after infection. Mice ovaries were collected for the further experiment. All animal experiments are in compliance with the ethical requirements of laboratory animals and laboratory animal management regulations of the Huai'an Second People's Hospital.

2.13 Immunohistochemistry assay

The Bax protein expression of mice ovaries was examined via immunohistochemistry. The ovarian tissue was taken for sectioning, deparaffinization, and hydration. After washing 3 times with PBS for 5 min each time, 3% H_2O_2 were added in the ovarian tissue to block endogenous peroxidase for 10 min at room temperature. Then Bax antibody (Wuhan Boster Biological Technology., Ltd, China) were added in the tissue after washing with PBS (3 times, 5 min each) and incubated overnight at 4 °C. Next, peroxidase-labeled goat anti-rabbit immunoglobulin (Ig) G secondary antibody (Wuhan Boster Biological Technology., Ltd, China) were added. Finally, DAB staining was conducted, and the results were observed using a microscope.

2.14 Statistical analysis

SPSS 21.0 software was utilized for statistical analysis. The data were expressed as mean ± standard deviation. The differences between multiple groups were compared with one-way ANOVA followed by LSD multiple comparisons. $P<0.05$ was considered statistically significant.

3 Results

3.1 The general characteristics and hormone levels of patients

Compared with the no-PCOS group, patients' BMI increases significantly in the PCOS group. Additionally, the PCOS group has significantly higher serum LH and T levels than the no-PCOS group. No statistically significant difference was seen in age and FSH levels between two groups in Table 1.

3.2 The expression of circ_0058063 is elevated in PCOS ovarian granulosa cells and follicular fluid

As presented in Figure 1A and 1B, qRT-PCR results indicate that the circ_0058063 level in ovarian granulosa cells and follicular fluid is remarkably higher in the PCOS group than the no-PCOS group. Additionally, the si-circ_0058063 group has a lower relative circ_0058063 expression, compared with the si-NC group in Figure 1C. These outcomes reveal that the expression of circ_0058063 is elevated in PCOS ovarian granulosa cells and follicular fluid.

3.3 Silencing circ_0058063 increases the levels of Aromatase mRNA, E2 and P4 in PCOS ovarian granulosa cells

We use qRT-PCR to detect the Aromatase mRNA expression in ovarian granulosa cells. Additionally, ELISA assay is applied to assess P4 and E2 levels in ovarian granulosa cells. As can be seen from Figure 2A-C, the levels of Aromatase mRNA, E2, and P4 are significantly increased in PCOS ovarian granulosa cells after silencing circ_0058063, compared with the si-NC group.

3.4 Silencing circ_0058063 enhances cell proliferation in PCOS ovarian granulosa cells

Cell proliferation is measured by colony formation assay and CCK-8 assay. As demonstrated in Figure 3A-C, the si-circ_0058063 group has remarkably higher ovarian granulosa cell proliferation than the si-NC group. In addition, western blot analysis is utilized to evaluate the Cyclin A and Cyclin D1 proliferation protein levels in each group. As displayed in Figure 3D and 3E, the protein levels of Cyclin A and Cyclin D1 in the si- circ_0058063 group increase significantly compared with those in the si-NC group. These results suggest that silencing circ_0058063 enhances cell proliferation in PCOS ovarian granulosa cells.

3.5 Silencing circ_0058063 inhibits apoptosis of PCOS ovarian granulosa cells

As presented in Figure 4A and 4B, the results of flow cytometry suggest that cell apoptosis is significantly decreased after silencing circ_0058063 in PCOS ovarian granulosa cells, compared with the si-NC group. In Figure 4C and 4D, western blot analysis indicates that silencing circ_0058063 significantly increases the expression of Bcl-2 protein and reduces the expression of Bax protein in the si-circ_0058063 group

than in the si-NC group. These data show that silencing circ_0058063 inhibits apoptosis of PCOS ovarian granulosa cells.

3.6 Knocking circ_0058063 improves ovary injury of PCOS mice

As presented in Figure 5A and 5B, the results of immunohistochemistry assay revealed that the rate of relative Bax positive cells is significantly decreased in the si-circ_0058063-1 group and si-circ_0058063-2 group, compared with the si-NC group. These data indicate that silencing circ_0058063 reduces ovary injury of PCOS mice.

4 Discussion

PCOS is the main cause of anovulatory infertility, which affects 80% of patients with anovulatory infertility (15, 16). At present, the mechanism of ovarian injury and abnormal follicular development in PCOS patients is not fully understood. PCOS is often closely related to repeated spontaneous abortions in infertile patients (17). Therefore, we must pay attention to this group of women of childbearing age, who may suffer adverse consequences due to complications of PCOS.

Our study finds that the expression of circ_0058063 in ovarian granulosa cells and follicular fluid in the PCOS group is significantly up-regulated. Besides, silencing circ_0058063 increases the levels of Aromatase mRNA, E2, and P4 in PCOS ovarian granulosa cells. In addition, silencing circ_0058063 can promote the proliferation of ovarian granulosa cells in PCOS patients. Furthermore, the results of flow cytometry reveal that after silencing circ_0058063, the survival rate of PCOS ovarian granulocytes is significantly increased, and the apoptosis rate is significantly reduced, indicating that silencing circ_0058063 may play an inhibiting role in ovarian granulosa cell apoptosis and have a promoting effect on cell survival in PCOS.

Circ_0058063 is only been studied in esophageal squamous-cell carcinomas and bladder cancer (10, 18, 19). In the progression of bladder cancer, circRNA_0058063 serves as a ceRNA via sponging miR-486-3p/FOXP4 and miR-145-5p/CDK6 axis (10, 19).Circ_0058063 stimulates glucose-uptake and increases the expression of GLUT1 in esophageal squamous-cell carcinomas (18). To the best of our knowledge, the present study is the first to investigate the function of circ_0058063 in PCOS.

5 Conclusions

In conclusion, silencing circ_0058063 enhances proliferation and inhibits apoptosis in PCOS ovarian granulosa cells. Such findings may offer vital insights into a therapeutic target for PCOS.

List Of Abbreviations

Polycystic ovary syndrome (PCOS), follicle stimulating hormone (FSH), testosterone (T), luteinizing hormone (LH), progesterone (P4) , estradiol (E_2), circular RNAs (circRNAs), in vitro fertilization and embryo

transfer (IVF-ET), body mass index (BMI), negative control (NC), optical density (OD).

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Huai'an Second People's Hospital. All patients voluntarily participated in this research project and signed informed consent. Moreover, all animal experiments are in compliance with the ethical requirements of laboratory animals and laboratory animal management regulations of the Huai'an Second People's Hospital.

Consent for publication

Not applicable.

Availability of data and material

Data available on request from the authors.

Competing interests

The authors confirm that there are no conflicts of interest.

Funding

Not applicable.

Authors' contributions

Chengcai Wen and Li Zhang designed the experiments, conducted the experiments and wrote the manuscript. All of the authors gave final approval to the submitted version of the manuscript.

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Table

Table 1. The general characteristics and hormone levels of patients in this study

Parameter	PCOS (N=9)	No-PCOS (N=9)
Age (year)	29.38 ± 3.23	30.52 ± 2.98
BMI (kg/m2)	24.32 ± 5.83	20.73 ± 3.26*
FSH (IU/L)	5.89 ± 2.27	5.08 ± 2.62
LH (IU/L)	11.95 ± 6.35	3.38 ± 1.94*
T (μg/L)	0.84 ± 0.31	0.47 ± 0.28*

PCOS, polycystic ovary syndrome; BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; T, testosterone. *, $P < 0.05$

Figures

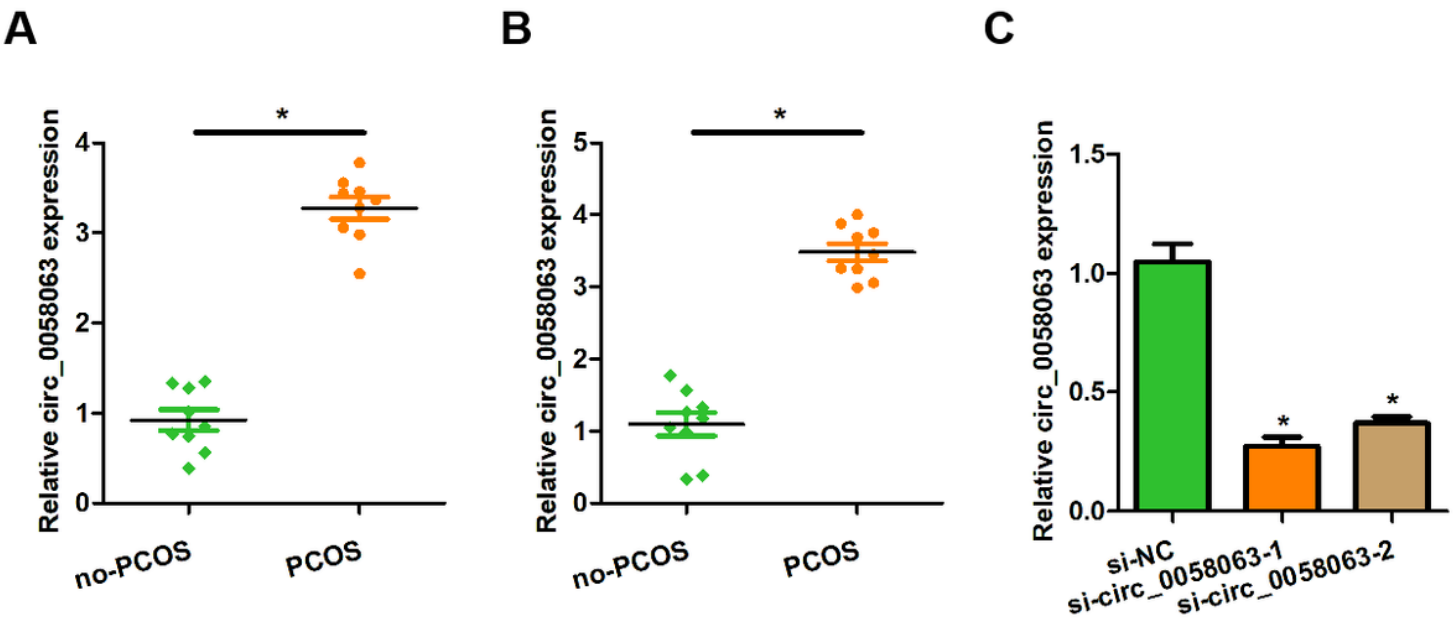

Figure 1

The expression of circ_0058063 is elevated in PCOS ovarian granulosa cells and follicular fluid (A) Expression of circ_0058063 in follicular fluid in two groups. (B) Relative circ_0058063 expression in

ovarian granulosa cells in two groups. (C) Expression of circ_0058063 in ovarian granulosa cells in three groups. PCOS, polycystic ovary syndrome; NC, negative control.*, P < 0.05

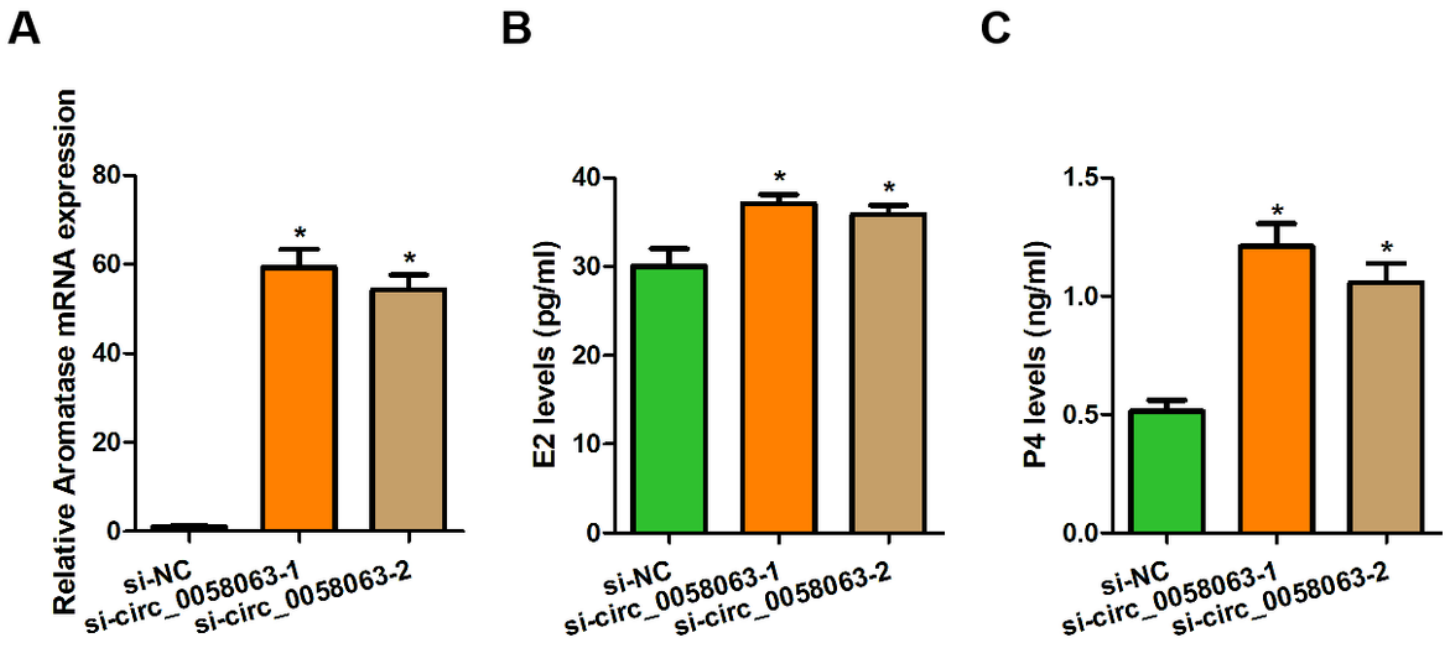

Figure 2

Silencing circ_0058063 increases the levels of Aromatase mRNA, E2 and P4 in PCOS ovarian granulosa cells (A) Expression of Aromatase mRNA in ovarian granulosa cells in each group. (B) Levels of E2 in ovarian granulosa cells in each group. (C) Levels of P4 in ovarian granulosa cells in each group. PCOS, polycystic ovary syndrome; E2, estradiol; P4, progesterone; NC, negative control.*, P < 0.05

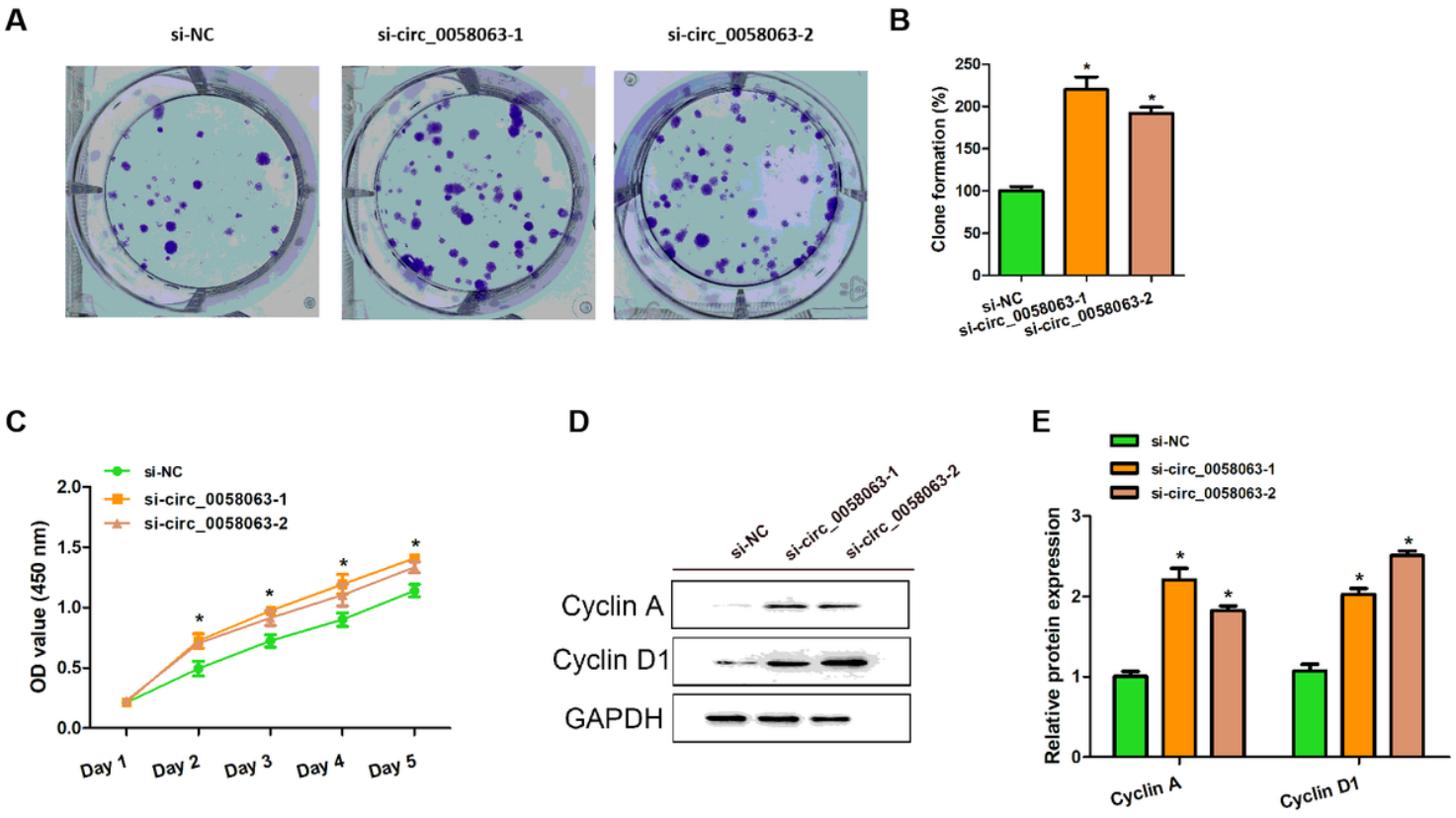

Figure 3

Silencing circ_0058063 enhances cell proliferation in PCOS ovarian granulosa cells (A) Cartogram of colony formation ability of PCOS ovarian granulosa cells in three groups. (B) Colony formation rate of PCOS ovarian granulosa cells in three groups. (C) Cell proliferation of PCOS ovarian granulosa cells in each group. (D, E) Relative protein expression of Cyclin A and Cyclin D1 in each group. NC, negative control, *, $P < 0.05$

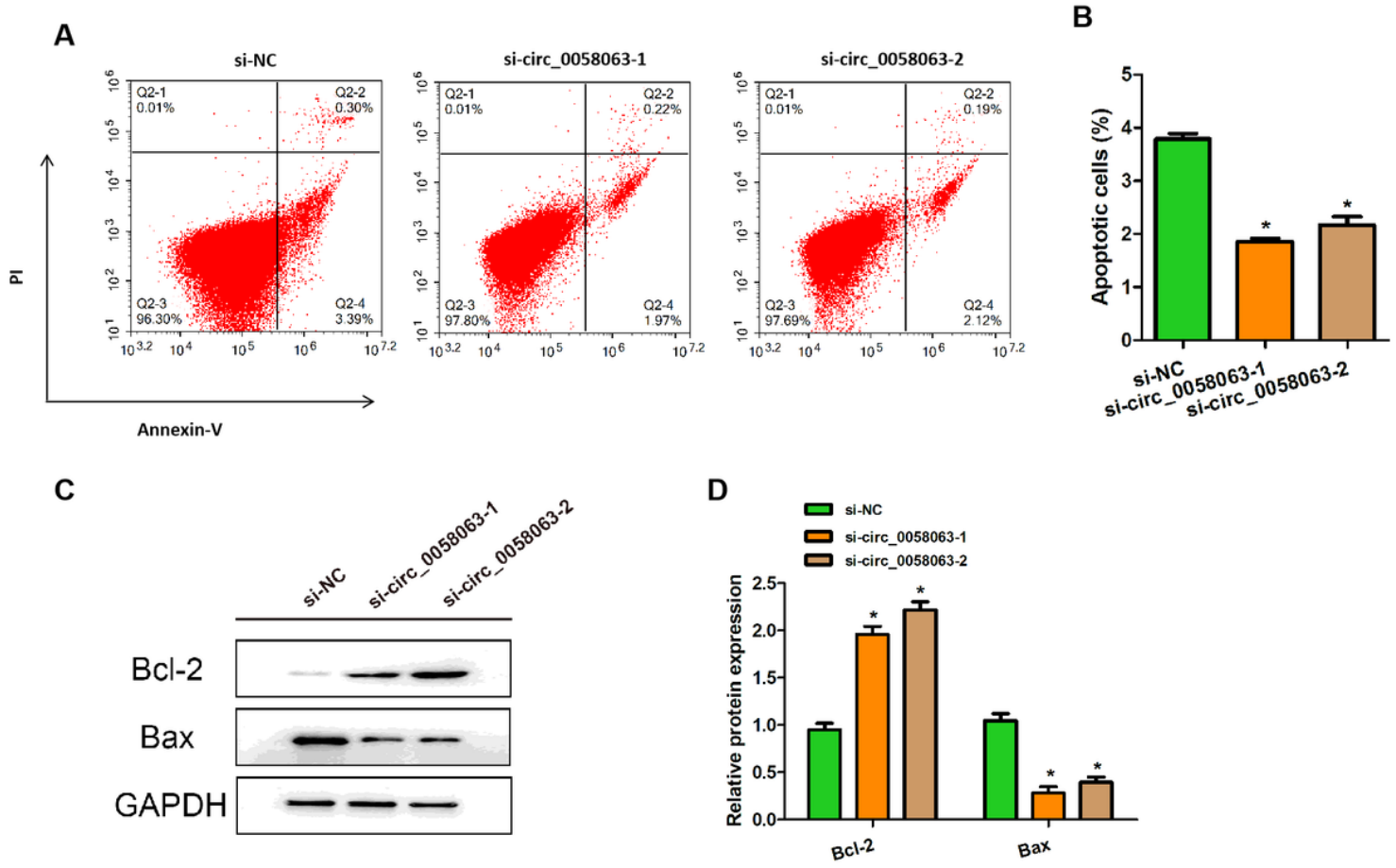

Figure 4

Silencing circ_0058063 inhibits apoptosis of PCOS ovarian granulosa cells (A, B) Cell apoptosis of PCOS ovarian granulosa cells was measured by flow cytometry assay; (C, D) Bcl-2 and BAX protein expression in each group. NC, negative control, *, P < 0.05

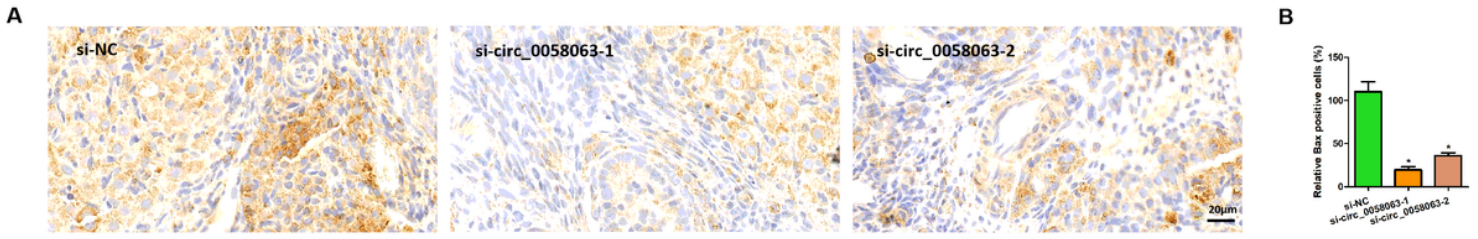

Figure 5

Knocking circ_0058063 improves ovary injury of PCOS mice (A) Representative images of immunohistochemistry assay of the ovary in each group. (B) Relative Bax positive cells in each group. NC, negative control, *, P < 0.05