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Proliferation of Ovarian Granulosa Cells in Polycystic Ovarian Syndrome Is Regulated by MicroRNA-24 by Targeting Wingless-Type Family Member 2B (WNT2B)

Authors' Contribution:

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Polycystic ovarian syndrome (PCOS) is a common endocrine disorder causing infertility among reproductive-age women. The molecular mechanisms underlying the development of PCOS are not well understood, and effective treatment options and therapeutic targets for PCOS are not available. This study was designed to investigate the role and therapeutic potential of miR-324 in PCOS.

Material/Methods: We used quantitative real time-polymerase chain reaction (qRT-PCR) to assess expression. Cell viability was determined by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay. Acridine orange/ethidium bromide (AO/EB) and annexin V/PI staining were performed to examine apoptosis. Western blot analysis was used to determine protein expression.

Results: Results showed that the expression of miR-324 was aberrantly and significantly downregulated in PCOS ovarian tissues and KGN ovarian granulosa cells. Nonetheless, ectopic expression of miR-324 expression inhibited the viability of KGN cells via induction of apoptotic cell death. *In silico* analysis showed Wingless-Type family member 2B (WNT2B) to be the target of miR-324, which was also validated by dual-luciferase reporter assay. We also found that the expression of WNT2B was upregulated in the KGN cells, and overexpression of miR-324 inhibited WNT2B expression. Similar to WNT2B overexpression, WNT2B silencing decreased the viability of the KGN. Furthermore, overexpression of WNT2B in KGN partially reversed the growth-inhibitory effects of miR-324 overexpression.

Conclusions: miR-324 regulates the proliferation of KGN cells in PCOs and be essential in the management of PCOS.

MeSH Keywords: **Apoptosis • Cell Migration Assays • Polycystic Ovary Syndrome**

Full-text PDF: <https://www.medscimonit.com/abstract/index/idArt/915320>



Background

Polycystic ovary syndrome (PCOS) is a prevalent endocrine disorder involving hyper-androgenism, insensitivity to insulin, and oligoovulation [1,2]. It affects approximately 10% of reproductive-age women, generally causing infertility [3]. PCOS is reported to increase the risk of type 2 diabetes [4]. Further, studies have shown that although women with mild PCOS tend to have normal ovulation, the disorder becomes chronic if untreated [5]. PCOS is also associated with increased androgen activity and often interferes with the biosynthesis of estrogen and progesterone. It has been reported that increased activity of androgen occurs in PCOS [6]. Although insulin-sensitizers such as metformin are used for the management of PCOS, such drugs have adverse effects that impair quality of life [7]. Therefore, research has focused on identifying the molecular mechanisms underlying PCOS to find the therapeutic targets for the complete treatment of PCOS. MicroRNAs (miRs) have been shown to have many roles in humans and have been reported to regulate the expression of 30% of human genes [8]. Around 20 nucleotides in length, miRs bind to the mRNAs of a wide array of human genes to enforce their transcriptional repression [9]. Studies directed at deciphering the roles of miRs have indicated that miR expression is often dysregulated under disease conditions; therefore, is believed that they may be involved in the onset and development of diseases such as cancer and diabetes [10]. A recent study indicated that miR-324 is aberrantly expressed in ovaries with of people with PCOS [11], but the exact role of miR-324 is yet to be established. The present study was therefore designed to examine the expression of miR-324 in normal and PCOS ovarian tissues and to assess the role of miR-324 in the KGN cell line obtained from PCOS ovaries. Our results show that the expression of miR-324 was significantly downregulated in the PCOS ovaries and in the KGN ovarian granulosa cell line. Nonetheless, ectopic expression of miR-324 in KGN cells significantly suppressed KGN cell proliferation via induction of apoptotic cell death. Moreover, *in silico* analysis and dual-luciferase assay showed it exerts its effects on ovarian granulosa cells in PCOS by targeting WNT2B. Hence, miR-324 may prove essential in the management of PCOS.

Material and Methods

Clinical samples and cell lines

After obtaining informed consent from all participants, PCOS and normal ovaries tissues were obtained from 15 women with PCOS and 15 normal women undergoing laparoscopy or hysterectomy at the Department of Obstetrics and Gynecology, Wuhan Children's Hospital (Wuhan Maternal and Child Healthcare Hospital), Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430015, China. The ovarian

granulosa KGN cell line and the normal ovarian cell line SV40 were obtained from the Cell Bank of Chinese Academy of Science (Shanghai, China) and maintained under standard conditions. The study was carried out in accordance with the Quality and Transparency of Health Research (EQUATOR) network guidelines [12]. The study was also approved by the Institutional Ethics committee under approval number 005/HU/37A/2018.

cDNA synthesis, qRT-PCR, and transfections

RNA was isolated from the tissues and cell lines using RNeasy kits, and cDNA was synthesized with an Omniscript RT (Qiagen, Inc.) kit from 2 μg of the RNA. The cycling conditions were as follows: 95°C for 20 s, followed by 40 cycles of 95°C for 15 s, and 58°C for 1 min. The expression was estimated by $2^{-\Delta\Delta\text{Ct}}$ method and actin was used as an internal control [13,14]. The transfection of KGN with different cell constructs was carried out using Lipofectamine 2000 reagent as per the manufacturer's guidelines.

MTT cell viability assay

For assessment of cell viability, KGN was transfected with appropriate constructs and incubated for 24 h at 37°C and then incubated with MTT (500 $\mu\text{g}/\text{mL}$) for 4 h. SDS (10%) was then added to dissolve the blue formazan formed. Finally, the optical density was assessed at 570 nm with a spectrophotometer (BD Biosciences, San Jose, CA, USA) and cell viability was determined as the percentage of the control.

AO/EB staining assay

The transfected KGN cells (0.6×10^6) were cultured in 6-well plates for 24 h at 37°C. We placed 25 μl of cell culture onto a glass slide and stained it with a 1- μL solution of AO/EB. The slides were covered with cover slips and examined under a fluorescence microscope.

Annexin V/PI staining assay

An ApoScan kit was used to determine the apoptotic KGN cell percentage. In brief, the transfected KGN cells (5×10^5 cells per well) were incubated at 37°C for 24 h, followed by staining with annexin V-FITC or PI. The percentage of apoptotic KGN cells at each concentration was then determined by flow cytometry.

Target identification and dual luciferase assay

The miR-324 target was identified by TargetScan version 7.2 (<http://www.targetscan.org>). For the luciferase reporter assay, the binding sites of the wild-type (WT) and the mutated (MUT) WNT2B 3'-UTR were cloned into the PGL3-REPORT luciferase vector. The KGN cells were co-transfected with the WT and MUT WNT2B UTR vectors and the miR-324 mimics. The luciferase

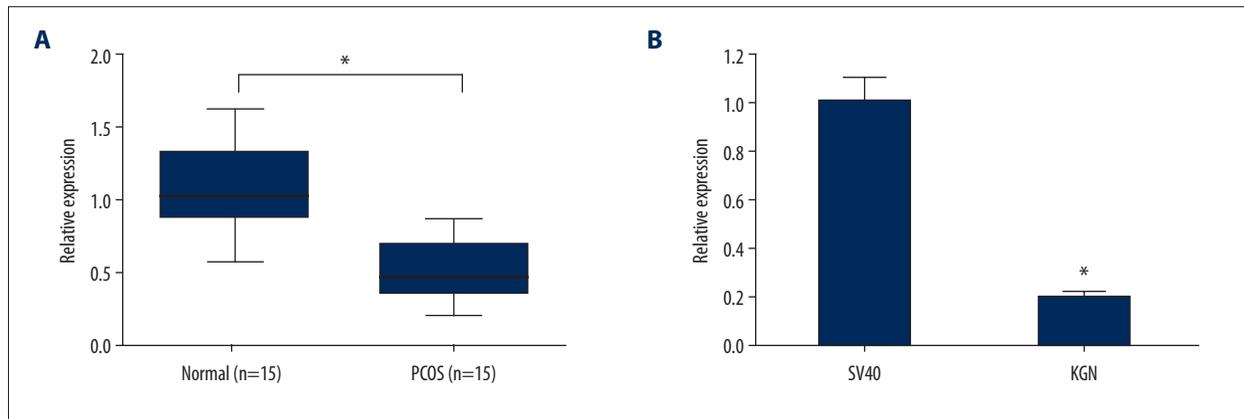


Figure 1. Expression of miR-324 in (A) the ovarian tissues of PCOS and normal women (n=15 for each) and (B) in ovarian KGN granulosa and SV40 normal ovarian cells as determined by qRT-PCR. The experiments were performed in triplicate and the values represent mean \pm SD (* $P < 0.05$).

activity was estimated using a luciferase reporter assay kit as per the guidelines of the supplier. Renilla luciferase activity was used to normalize the data.

Western blotting

The transfected KGN cells were harvested by centrifugation. The cells were then lysed in lysis buffer containing the protease inhibitor. About 45 μ g of proteins from each sample were subjected to 10% separation, followed by transfer to a polyvinylidene difluoride (PVDF) membrane. Next, fat-free milk was used to block the membrane at room temperature for 1 h. Thereafter, the membranes were treated with primary antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C overnight. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Finally, the signal was detected using the Odyssey Infrared Imaging System. Actin was used as control for normalization.

Statistical analysis

The experiments were performed in triplicate and the values represent the mean results of experiments carried out in triplicate \pm SD. One-way ANOVA followed by Tukey's test with GraphPad prism 7 software was employed for statistical analysis. $p < 0.05$ was taken to indicative a statistically significant difference.

Results

miR-324 is significantly downregulated in PCOS ovaries and KGN cells

The miR-324 expression was examined in normal and PCOS ovary tissues by qRT-PCR (Figure 1A). The results showed that

miR-324 exhibited aberrant expression and was significantly downregulated ($p < 0.05$) in all PCOS ovary tissues (Figure 1). The expression of miR-324 was more than 2-fold lower in the PCOS tissues relative to the normal ovarian tissues. The miR-324 expression was also examined in KGN ovarian granulosa cells and normal SV40 ovarian cells. The results showed that the expression of miR-12b was about 9-fold lower in the KGN cells relative to the normal SV40 cells (Figure 1B).

Overexpression of miR-324 KGN Cells triggers apoptosis

Next, we assessed the role of miR-324 in PCOS by transfecting KGN cells with either the miR-324 mimics or NC (negative control). The overexpression of miR-324 in KGN cells was validated by RT-PCR, showing that transfection of the KGN cells with miR-324 mimics resulted in a 6.2-fold increase in the miR-324 expression relative to NC-transfected cells (Figure 2A). Then, the proliferation rate of the NC- and miR-324 mimics-transfected KGN cells was determined by MTT assay, showing that the transfection of KGN cells with miR-324 mimics significantly decreased cell viability (Figure 2B). To understand the mechanism of action of miR-324, AO/EB staining was also performed to examine if miR-324 overexpression triggers apoptosis in the KGN cells. AO/EB staining revealed that miR-324 overexpression activated apoptotic cell death in the KGN cells transfected with miR-324 mimics (Figure 2C). Annexin V/PI staining showed that the percentage of apoptotic cells was 28% in miR-324 mimics-transfected KGN (Figure 2D). We also performed Western blot analysis of the NC- and miR-324-transfected KGN cells, showing that miR-324 overexpression in KGN cells caused upregulation of Bax and cleaved caspase-3 and decrease of Bcl-2 (Figure 3). These results clearly indicate that overexpression of miR-324 inhibits the proliferation of the KGN cells by induction of apoptosis.

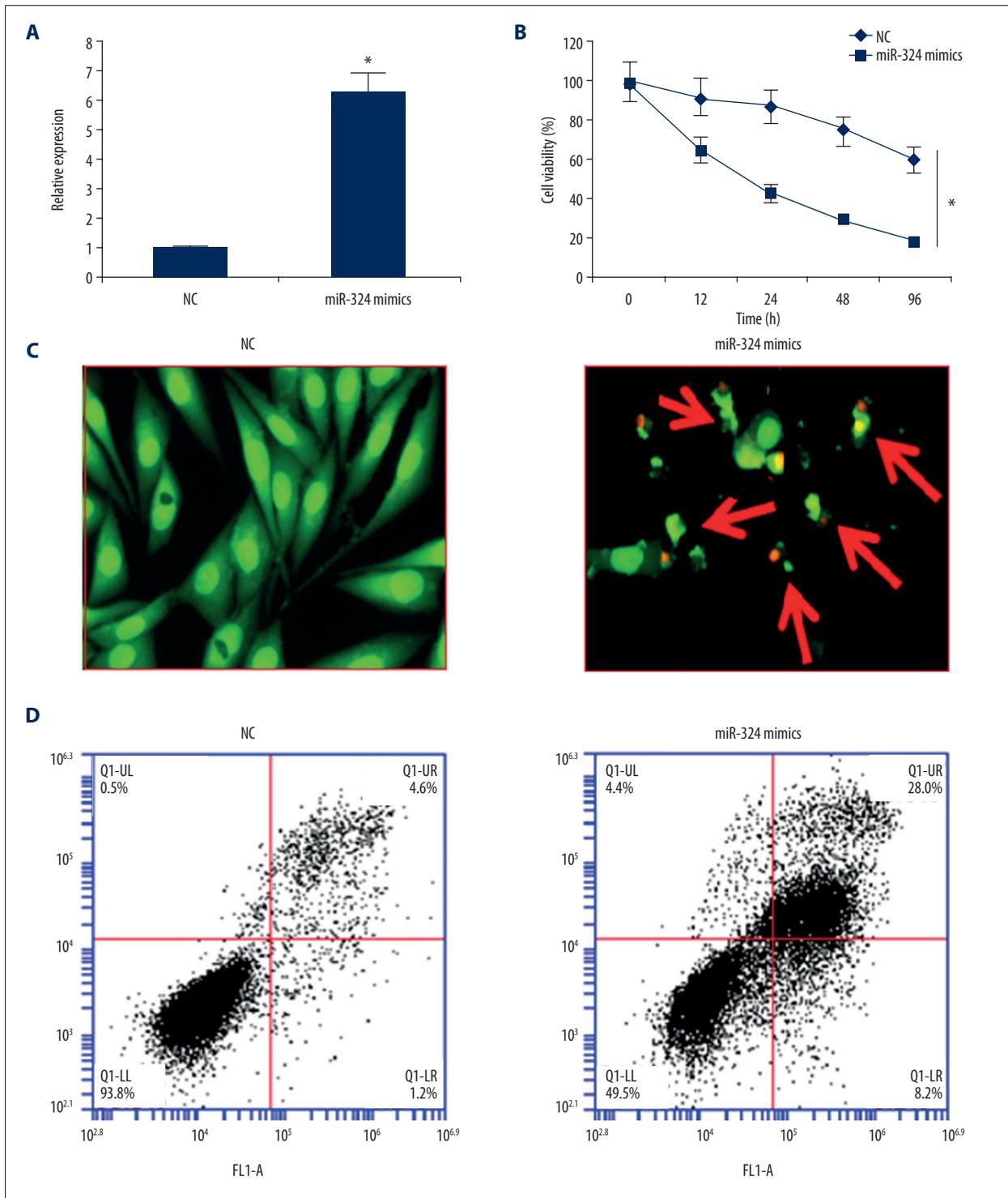


Figure 2. Overexpression of miR-324 triggers apoptosis in KGN ovarian granulosa cells of PCOS, (A) expression of miR-324 in NC- and miR-324 mimics-transfected KNG cells, (B) Cell viability of the miR-324 in NC- and miR-324 mimics-transfected KNG cells, (C) AO/EB staining of miR-324 in NC and miR-324 mimics-transfected KNG cells and (D) Annexin V/PI staining of miR-324 in NC- and miR-324 mimics-transfected KNG cells. The experiments were performed in triplicate and the values represent mean \pm SD (* P<0.05).

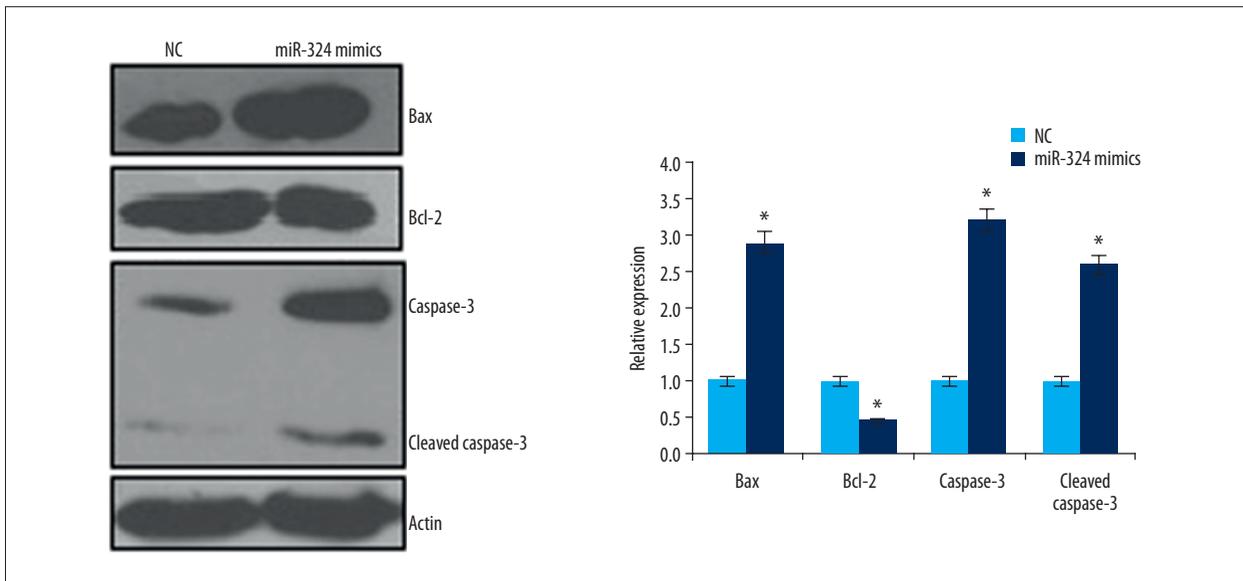


Figure 3. Expression of apoptosis related proteins in the NC- and miR-324 mimics-transfected KNG cells as determined by Western blot analysis. The experiments were performed in triplicate and the values represent mean \pm SD (* $P < 0.05$).

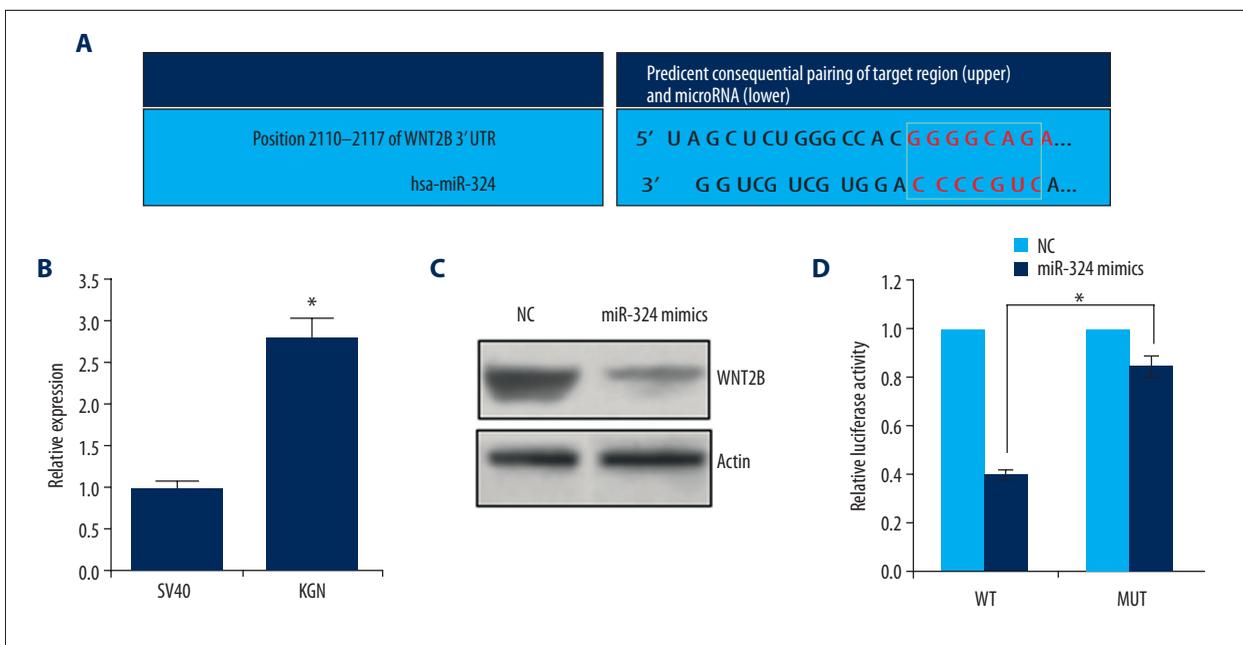


Figure 4. miR-324 exerts its effects by targeting WNT2B. (A) TargetScan analysis showing WNT2B of miR-324, (B) Expression of WNT2B in the KGN and SV40 cells, (C) Expression of WNT2B in NC and miR-324 mimics transfected KNG cells, and (D) dual-luciferase assay. The experiments were performed in triplicate and the values represent mean \pm SD (* $P < 0.05$).

miR-324 targets WNT2B in KGN cells

TargetScan analysis of miR-324 was performed to identify the target of miR-324 in KGN ovarian granulosa cells, and WNT2B was identified as the potential target of miR-324 in KGN ovarian granulosa cells (Figure 4A). Therefore, the expression of WNT2B was evaluated in the NC- and miR-324-transfected KGN cells, which revealed that the expression of WNT2B was

significantly upregulated in KGN (Figure 4B). Nonetheless, Western blot analysis showed that overexpression of miR-324 in KGN cells decreased the expression of WNT2B (Figure 4C). Dual-luciferase assay was also performed, which further validated WNT2B as the target of miR-324 (Figure 4D).

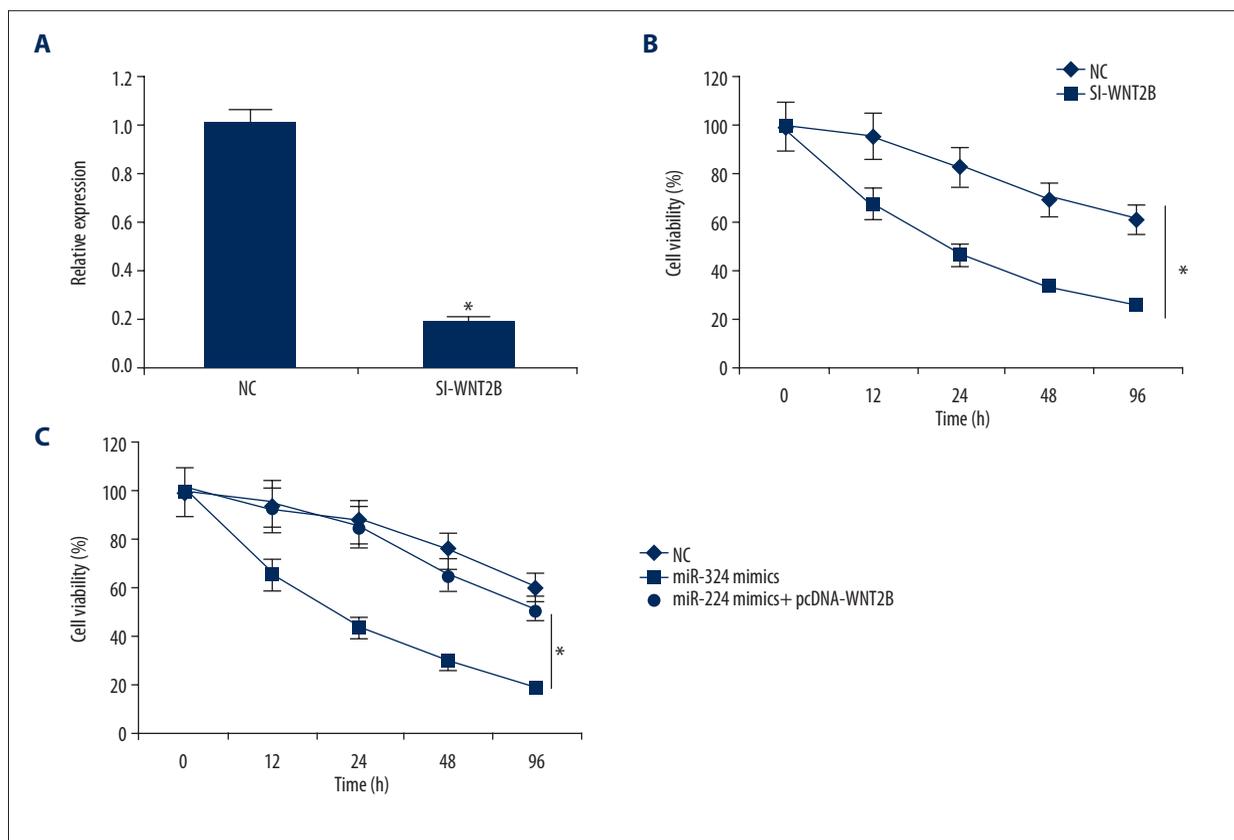


Figure 5. Silencing of WNT2B in KGN cells inhibits their proliferation. (A) Expression of WNT2B in the NC- and Si-WNT2B-transfected KGN cells, (B) Cell viability of the NC- and Si-WNT2B-transfected KGN cells, and (C) Overexpression of WNT2B in KGN cells partially reversed the effects of miR-324 overexpression on cell viability. The experiments were performed in triplicate and the values represent mean \pm SD (* $P < 0.05$).

WNT2B partially recues the effects of miR-324 overexpression on KGN cell proliferation

To understand the effects of WNT2B silencing on the proliferation of ovarian granulosa KGN cells, the GN cells were transfected with NC or Si-WNT2B and the silencing of WNT2B expression was validated by qRT-PCR analysis, showing a 9-fold decrease in WNT2B expression in Si-WNT2B-transfected cells relative to the NC-transfected KGN cells (Figure 5A). MTT showed that Si-WNT2B silencing caused a significant ($p < 0.05$) decrease in the viability of the KGN cells (Figure 5B). These effects were almost identical to that of miR-324 overexpression.

We also assessed the effects of WNT2B overexpression on the miR-324 mimics-transfected KGN cells, showing that WNT2B overexpression at least partially reversed the effects of miR-324 overexpression on the viability of KGN cells, which suggests that WNT2B is directly involved in the miR-324-mediated effects (Figure 5C).

Discussion

PCOS is a frequently diagnosed metabolic and endocrine abnormality and is one of the causes for infertility in woman [15]. Although researchers have sought to understand the molecular mechanisms underlying PCOS, the explicit cause of PCOS development is unclear. PCOS has been observed to be allied with changes in gonadotropin, enhancement of steroidogenic enzymes, insensitivity to insulin, and oligoovulation, as well as irregular menstruation, which in turn results in sub-fertility [16]. Dietary restriction and fasting have recently become popular to modify metabolism, even achieving durable epigenetic modulation. When fasting, the metabolic imbalance typical of PCOS may improve significantly [17–19]. Accumulating evidence suggests that one of the most important mechanisms in PCOS pathogenesis is insulin-resistance; therefore, the efficacy of insulin-sensitizers such as inositol isoforms has gained increasing attention [20,21]. In addition, insulin-sensitizers like metformin are also utilized for the management of PCOS. However, these treatments are associated with a number of adverse effects. Moreover, PCOS has also been found to increase the risk of cancer development due to unrelenting

stimulation of endometrial tissue by estrogen [22]. It is imperative to explore treatment options for better management of PCOS. Over the last few decades, miRs have been considered to be important therapeutic targets for the management of deadly diseases [3]. Many miRs have been found to be dysregulated in PCOS ovaries. A previous study found that miR-125b regulates the proliferation of ovarian granulosa cells in PCOS [22], and another study reported that miR-483 is down-regulated in polycystic ovarian syndrome and inhibits KGN cell proliferation [23]. Similarly, the expression of miR-324 has been shown to be dysregulated in PCOS ovaries [11]. Consistently, we observed that miR-324 is aberrantly downregulated in PCOS ovaries and in the ovarian granulosa KGN cell lines. Ectopic expression of miR-324 expression causes suppression of the proliferation of KGN cells by triggering apoptotic cell death of the KGN cells, which was also allied with increased expression of Bax and cleaved caspase 3. In addition, *in silico* analysis showed that miR-324 exerts its effects

by targeting WNT2B. WNT2B is a member the WNT-family and has been shown to be involved in several development-related processes in *Drosophila* flies [24]. WNT2B has been reported to play a role in the development and progression of human cancers [25]. Here, we showed that WNT2B silencing led to inhibition of proliferation of KGN cells, similar to the effect of miR-324 overexpression. Furthermore, it was observed that overexpression of WNT2B partially reversed the effects of miR-324 overexpression on the proliferation of the KGN cells.

Conclusions

We found that miR-324 inhibits the proliferation of ovarian granulosa cells in PCOS by targeting WNT2B and as such may serve as an important therapeutic target for the management of PCOS. However, further studies are required to establish miR-324 as a therapeutic target for PCOS.

References:

1. Sen A, Prizant H, Light A et al: Androgens regulate ovarian follicular development by increasing follicle stimulating hormone receptor and microRNA-125b expression. *Proc Natl Acad Sci USA*, 2014; 111(8): 3008–13
2. Azziz R, Carmina E, Chen Z et al: Polycystic ovary syndrome. *Nat Rev Dis Primers*, 2016; 2: 16057
3. Knochenhauer ES, Key TJ, Kahsar-Miller M et al: Prevalence of the polycystic ovary syndrome in unselected black and white women of the McCartney CR, Marshall JC. *Polycystic ovary syndrome*. *N Engl J Med*, 2016; 375(1): 54–64
4. Sirmans SM, Pate KA: Epidemiology, diagnosis, and management of polycystic ovary syndrome. *Clin Epidemiol*, 2014; 6: 1–7
5. Ehrmann DA, Barnes RB, Rosenfield RL et al: Prevalence of impaired glucose tolerance and diabetes in women with polycystic ovary syndrome. *Diabetes Care*, 1999; 22(1): 141–46
6. Naderpoor N, Shorakae S, de Courten B et al: Metformin and lifestyle modification in polycystic ovary syndrome: systematic review and meta-analysis. *Hum Reprod Update*, 2015; 21(5): 560–74
7. Lo JC, Yang J, Gunderson EP et al: Risk of type 2 Diabetes Mellitus following Gestational Diabetes Pregnancy in Women with Polycystic Ovary Syndrome. *J Diabetes Res*, 2017; 1: 20–27
8. Croce CM, Calin GA: miRNAs, cancer, and stem cell division. *Cell*, 2005; 122(1): 6–7
9. Carthew RW, Sontheimer EJ: Origins and mechanisms of miRNAs and siRNAs. *Cell*, 2009; 136(4): 642–55
10. Slaby O, Svoboda M, Fabian P et al: Altered expression of miR-21, miR-31, miR-143 and miR-145 is related to clinicopathologic features of colorectal cancer. *Oncology*, 2007; 72(5–6): 397–402
11. Jiang YC, Ma JX: The role of MiR-324-3p in polycystic ovary syndrome (PCOS) via targeting WNT2B. *Eur Rev Med Pharmacol Sci*, 2018; 22(11): 3286–93
12. Altman DG, Simera I, Hoey J et al: EQUATOR: Reporting guidelines for health research. *Lancet*, 2008; 371(9619): 1149–50
13. Steibel JP, Poletto R, Coussens PM et al: A powerful and flexible linear mixed model framework for the analysis of relative quantification RT-PCR data. *Genomics*, 2009; 94(2): 146–52
14. Hua F, Li CH, Chen XG et al: Daidzein exerts anticancer activity towards SKOV3 human ovarian cancer cells by inducing apoptosis and cell cycle arrest, and inhibiting the Raf/MEK/ERK cascade. *Int J Mol Med*, 2018; 41(6): 3485–92
15. Escobar-Morreale HF, Roldán-Martín MB et al: Type 1 diabetes and polycystic ovary syndrome: Systematic review and meta-analysis. *Diabetes Care*, 2016; 39(4): 639–48
16. Murri M, Insenser M, Fernández-Durán E et al: Non-targeted profiling of circulating microRNAs in women with polycystic ovary syndrome (PCOS): Effects of obesity and sex hormones. *Metabolism*, 2018; 1: 6–12
17. Laganà AS, Rossetti P, Sapia F et al: Evidence-based and patient-oriented inositol treatment in polycystic ovary syndrome: Changing the perspective of the disease. *Int J Endocrinol Metab*, 2017; 15: e43695
18. Laganà AS, Vitale SG, Noventa M, Vitagliano A: Current management of polycystic ovary syndrome: From bench to bedside. *Int J Endocrinol*, 2018; 1: 1–4
19. Laganà AS, Vitagliano A, Noventa M et al: Myo-inositol supplementation reduces the amount of gonadotropins and length of ovarian stimulation in women undergoing IVF: A systematic review and meta-analysis of randomized controlled trials. *Arch Gynecol Obstet*, 2018; 4: 1–2
20. Chiofalo B, Laganà AS, Palmara V et al: Fasting as possible complementary approach for polycystic ovary syndrome: Hope or hype? *Med Hypotheses*, 2017; 105: 1–3
21. Akbar MF, MedSc M, Rizkinov M, Jumsa MD: The impact of ramadhan fasting in women with PCOS: An association between FPG and lipid profiles. *Arch Iranian Med*, 2016; 19(4): 308
22. Adams J, Polson DW, Franks S: Prevalence of polycystic ovaries in women with anovulation and idiopathic hirsutism. *Br Med J (Clin Res Ed)*, 1986; 293: 355–59
23. Sen A, Prizant H, Light A et al: Androgens regulate ovarian follicular development by increasing follicle stimulating hormone receptor and microRNA-125b expression. *Proc Natl Acad Sci USA*, 2014; 111(8): 3008–13
24. Xiang Y, Song Y, Li Y et al: miR-483 is down-regulated in polycystic ovary syndrome and inhibits kgn cell proliferation via targeting insulin-like growth factor 1 (IGF1). *Med Sci Monit*, 2016; 22: 3383–93
25. Katoh M: Differential regulation of WNT2 and WNT2B expression in human cancer. *Int J Mol Med*, 2001; 8(6): 657–60
26. Wang H, Fan L, Xia X et al: Silencing Wnt2B by siRNA interference inhibits metastasis and enhances chemotherapy sensitivity in ovarian cancer. *Int J Gynecol Cancer*, 2012; 22(5): 755–61