

RESEARCH ARTICLE

miR-221-3p regulates apoptosis of ovarian granulosa cells via targeting FOXO1 in older women with diminished ovarian reserve (DOR)

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Abstract

In our earlier study, we showed that the expression of microRNA-221-3p (miR-221-3p) was significantly lower in women of advanced age with diminished ovarian reserve (DOR) compared with young women with normal ovarian reserve (NOR). Therefore, in this study, we aimed to explore how miR-221-3p regulates apoptosis of granulosa cells and the pathogenesis of DOR. Bioinformatics prediction and dual-luciferase reporter assay were conducted to identify the target gene of miR-221-3p. miR-221-3p expression was manipulated by transfecting KGN cells with miR-221-3p mimics, inhibitor, and negative control. Following transfection, apoptosis of granulosa cells was determined by flow cytometry, and the expression of the target gene was measured by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot analysis (WB). In addition, the expression of the target gene in granulosa cells of DOR patients and NOR patients was measured. miR-221-3p were found to directly bind the 3' untranslated region of Forkhead box O1 (*FOXO1*). Transfection with miR-221-3p mimics significantly decreased the apoptosis rate of KGN cells compared with transfection with miR-221-3p inhibitors. The expression level of miR-221-3p was negatively correlated with the messenger RNA and protein levels of the *FOXO1* gene. Besides, *FOXO1* expression was upregulated in DOR patients. In conclusion, these results provide evidence that downregulation of miR-221-3p expression promotes apoptosis of granulosa cells by upregulating *FOXO1* expression, thus serving an important role in DOR pathogenesis.

KEYWORDS

apoptosis, diminished ovarian reserve, FOXO1, miR-221-3p

1 | INTRODUCTION

Diminished ovarian reserve (DOR) refers to a decrease in the number and quality of oocytes and clinically manifests as hypomenorrhea, oligomenorrhea, amenorrhea, and infertility. Approximately 10% of women

seeking fertility treatment are diagnosed with DOR (Y. Wang et al., 2016). In 2011, the prevalence of DOR in the United States was reported to be 26%, having increased from 19% in 2004 (Devine et al., 2015). In China, with the implementation of the two-child policy, as well as the delay in childbearing, more women of advanced age are seeking fertility

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treatment. DOR significantly affects the physical and psychological health and reduce the quality of life, especially for women who are desperate to have a baby. Currently, the main treatment of DOR is mainly based on hormone replacement therapy, however, its efficacy has not been comprehensively investigated. Even with assisted reproductive technology, this population of women still experience a lower pregnancy rate due to the small quantity and low-quality of eggs extracted. The mutual communication between oocytes and granulosa cells (GCs) is crucial for oocytes development and maturation (Komatsu & Masubuchi, 2018). Apoptosis of GCs, which lead to follicular atresia, is an important pathomechanism of DOR (Murase et al., 2018). Although numerous studies have been performed on DOR, its pathogenesis remains unclear.

MicroRNAs (miRNAs) are noncoding RNAs comprising of about 19–25 nucleotides (Kittlmann & McGregor, 2019; Tang et al., 2019). They regulate gene expression by interacting with the 3'-untranslated region (3'-UTR) of target messenger RNAs (mRNAs). Mounting evidence suggests that miRNAs modulate follicular growth and development, GCs proliferation, apoptosis, differentiation, and sex hormone secretion (Andreas et al., 2016; J. Liu et al., 2018; M. Wang et al., 2017; Yao et al., 2018).

Apoptosis of ovarian GCs is an important cause of DOR and has consistently formed the research focus in the etiology of DOR. During in vitro fertilization or intracytoplasmic sperm injection in humans, a high rate of GCs' apoptosis is related to empty follicles, low number of eggs obtained, and low quality of oocytes and embryos (Almeida et al., 2018). In our previous study (D. Liu et al., 2020), we compared differentially expressed miRNAs in GCs between DOR patients of advanced age and young women with normal ovarian reserve (NOR). miR-221-3p was found to be significantly downregulated in DOR patients of advanced age. However, the role of miR-221-3p in the pathogenesis of DOR remains elusive. This study, explored whether miR-221-3p regulated apoptosis of GCs.

2 | RESULTS

2.1 | Basic characteristics of two patients

Results presented in Table 3 show the age, body mass index (BMI), the levels of basal follicle stimulating hormone (FSH), luteinizing hormone

TABLE 1 The combinations of transfected plasmids

Serial number	Plasmid combinations
1	MiRNA NC+FOXO1-WT
2	MiR-221-3p mimics+FOXO1-WT
3	MiRNA NC+FOXO1-MT
4	MiR-221-3p mimics+FOXO1-MT
5	MiRNA NC+BCL2L11-WT
6	MiR-221-3p mimics+BCL2L11-WT
7	MiRNA NC+BCL2L11-MT
8	MiR-221-3p mimics+BCL2L11-MT

TABLE 2 Primers used for quantitative real-time polymerase chain reaction

Gene	Primer	Primer sequence
MiR-221-3P	Primer F	5'-CGCGAGCTACATTGTCTGCTG-3'
	Primer R	5'-AGTGCAGGGTCCGAGGTATT-3'
U6	Primer F	5'-CTCGCTTCGGCAGCAC-3'
	Primer R	5'-AACGCTTCACGAATTTGCGT-3'
FOXO1	Primer F	5'-CAGACTTGGCAGCAAAGAC-3'
	Primer R	5'-AAGACATGAGGCCATCAC-3'
GAPDH	Primer F	5'-AATCCCATCACCATCTTC-3'
	Primer R	5'-AGGCTGTTGCATACTTC-3'

Abbreviations: FOXO1, Forkhead box transcription factor O1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

TABLE 3 The characteristics of patients with DOR or NOR

Characteristics	DOR (n = 20)	NOR (n = 20)	p value
Age, year	42.90 ± 4.10	29.60 ± 2.41	<.01
BMI, kg/m ²	22.28 ± 2.00	21.99 ± 1.86	.63
Basal FSH, IU/L	15.72 ± 4.51	6.94 ± 1.26	<.01
Basal LH, IU/L	4.64 ± 1.48	4.49 ± 1.85	.77
Basal E ₂ , pg/ml	39.63 ± 25.20	40.12 ± 19.40	.94
Basal P ₄ , ng/ml	0.59 ± 0.38	0.48 ± 0.18	.23
AMH, ng/ml	0.48 ± 0.29	5.81 ± 1.86	<.01
AFC, n	4.15 ± 1.53	19.7 ± 3.53	<.01

Abbreviations: AMH, anti-Mullerian hormone; AFC, antral follicle count; BMI, body mass index; E₂, estrogen; FSH, follicle stimulating hormone; LH, luteinizing hormone; P₄, progesterone.

(LH), estradiol (E₂), progesterone (P₄), anti-Mullerian hormone (AMH), antral follicle count (AFC) of 20 women with DOR, and 20 women with NOR. Notably, the BMI, LH, and E₂ were not significantly different between the two groups. However, patients with DOR (mean age: 42.90 ± 4.10) had significantly higher basal FSH level (15.72 ± 4.51 vs. 6.94 ± 1.26, *p* < .05), lower AMH level (0.48 ± 0.29 vs. 5.81 ± 1.86, *p* < .05), and lower AFC (4.15 ± 1.53 vs. 19.7 ± 3.53, *p* < .05) compared with NOR patients (mean age: 29.60 ± 2.41).

2.2 | FOXO1 gene is a downstream target of miR-221-3p but not BCL2L11

A total of 18 possible target genes of miR-221-3p were shown in Table 4. Only two target genes (*FOXO1* and *BCL2L11*), associated with apoptosis, were considered as our candidates. Using a bioinformatics software, we predicted specific binding sites between miR-221-3p and *FOXO1*, *BCL2L11*. A schematic drawing showing the binding sequences is shown in Figure 1a,b. Results of the dual-luciferase assay showed that miR-221-3p mimics significantly reduced the relative

TABLE 4 The list of target genes predicted for miR-221-3p

Target gene	Gene name
CAMK1D	Calcium/calmodulin-dependent protein kinase ID
NAA25	N(alpha)-acetyltransferase 25, NatB auxiliary subunit
FOXO1	Forkhead box O1
BCL2L11	BCL2-like 11 (apoptosis facilitator)
CACNB4	Calcium channel, voltage-dependent, beta 4 subunit
FRAT2	Frequently rearranged in advanced T-cell lymphomas 2
NGRN	Neugrin, neurite outgrowth associated
SPRED2	Sprouty-related, EVH1 domain containing 2
UBE2J1	Ubiquitin-conjugating enzyme E2, J1
HIPK2	Homeodomain interacting protein kinase 2
DGKH	Diacylglycerol kinase, eta
RPH3A	Rabphilin 3A homolog (mouse)
SHANK2	SH3 and multiple ankyrin repeat domains 2
CUX2	Cut-like homeobox 2
RND2	Rho family GTPase 2
AKAP13	A kinase (PRKA) anchor protein 13
C6orf120	Chromosome 6 open reading frame 120
SESN3	Sestrin 3

luciferase activity in the presence of FOXO1-WT, compared with the negative control (NC). However, when the mutation occurred in the binding site region of FOXO1 3'-UTR (FOXO1-MT), the suppressive effect of miR-221-3p mimics was abrogated (Figure 1c). Besides, a significant difference was found between the relative luciferase activity of cells transfected with BCL2L11-WT plus miR-221-3p mimics and BCL2L11-WT plus miRNA NC. These significant differences persisted even after induced mutation of BCL2L11 3'-UTR (BCL2L11-MT). Of note, miR-221-3p mimics upregulated the relative luciferase activity of the plasmid containing the BCL2L11-WT (Figure 1d). This indicated that miR-221-3p did not bind to the 3'-UTR of BCL2L11. Taken together, these results suggested that FOXO1, but not BCL2L11, was a target gene of miR-221-3p.

2.3 | miR-221-3p suppresses the apoptosis of KGN cells

To further determine the role of miR-221-3p in apoptosis, flow cytometry was conducted in KGN cells transfected with the miRNA NC (Figure 2a), miR-221-3p mimics (Figure 2b), and miR-221-3p inhibitor (Figure 2c). The results showed that cells treated with miR-221-3p mimics had a lower apoptosis rate compared with those treated miRNA NC ($p < .05$). Treatment with miR-221-3p inhibitor resulted in a higher apoptosis rate compared with miRNA NC treatment (Figure 2d). These results suggested that miR-221-3p inhibited apoptosis of KGN cells.

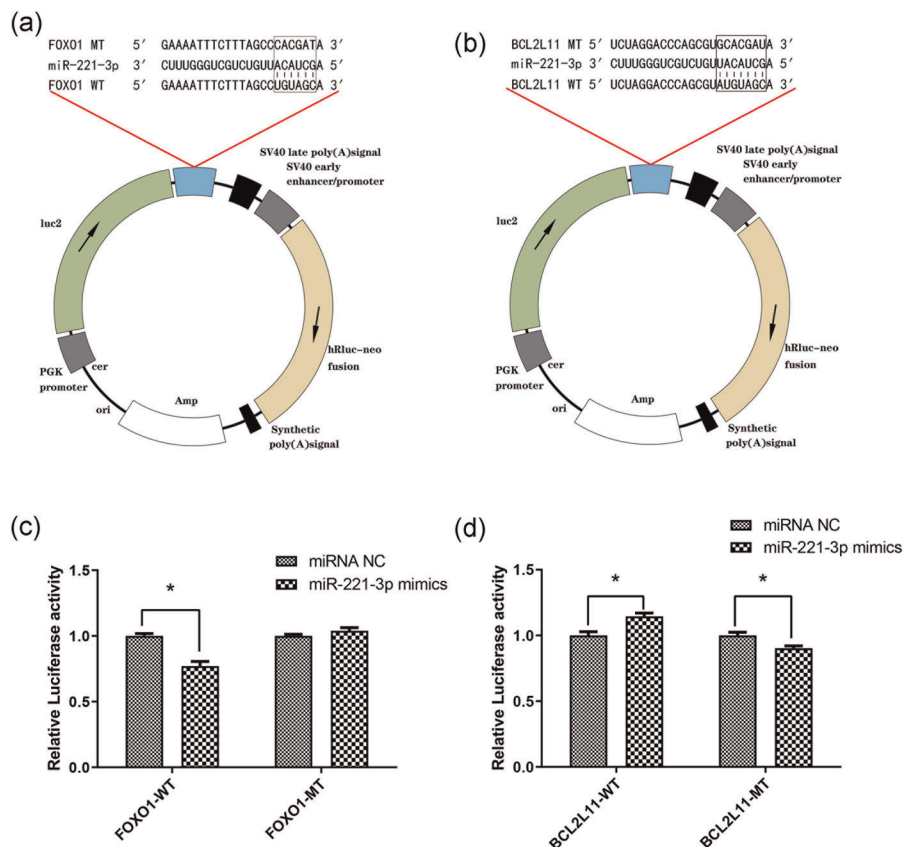


FIGURE 1 FOXO1, but not BCL2L11, was a target gene of miR-221-3p. The predicted binding sites and mutations in the binding sites of miR-221-3p in the 3'-UTR of FOXO1 (a) and BCL2L11 (b). Detection of direct interaction between miR-221-3p and FOXO1 (c), as well as miR-221-3p and BCL2L11 (d) by the dual luciferase assay. The experiments were performed in five replicate holes. FOXO1, Forkhead box transcription factor O1; BCL2L11, BCL2 Like 11. * $p < .05$. miR, microRNA; UTR, untranslated region

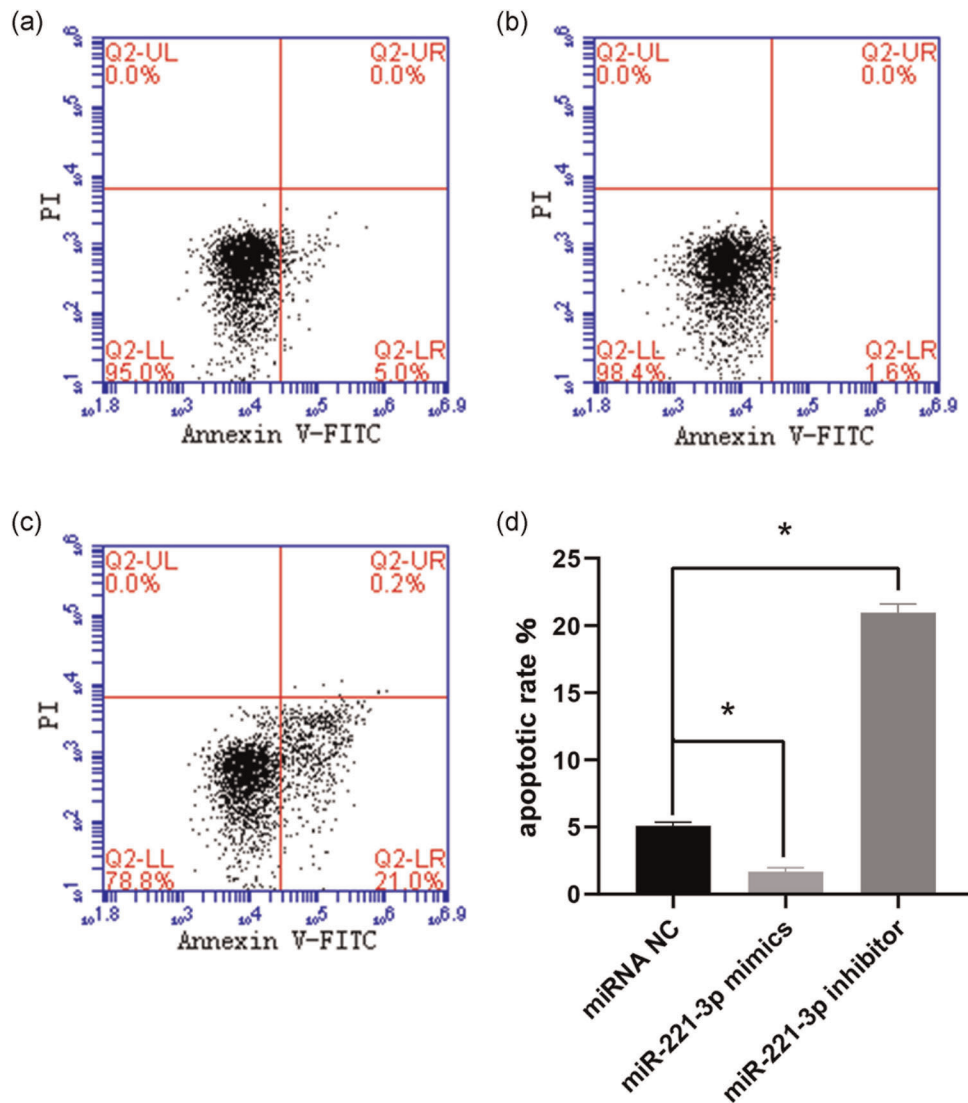


FIGURE 2 The effects of miR-221-3p on KGN cell apoptosis. Representative images of cell apoptosis in KGN cells after transfection with miRNA NC (a), miR-221-3p mimics (b), and miR-221-3p inhibitor (c). Histogram representation of apoptotic rate in KGN cells (d). The experiments were repeated three times and data are presented as means \pm SD ($n = 3$). * $p < .05$. miR, microRNA

2.4 | miR-221-3p expression level is negatively correlated with FOXO1 expression

We examined whether miR-221-3p expression influenced FOXO1 expression at both the mRNA and protein levels. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) results showed that miR-221-3p was significantly expressed in the mimics group and significantly suppressed in the inhibitor group compared with the negative control group (Figure 3a). This indicated a successful transfection in the aforementioned groups. Besides, FOXO1 mRNA level was significantly lower in cells transfected with miR-221-3p mimics compared with those transfected with miRNA NC. By contrast, transfection with the miR-221-3p inhibitor showed a significantly high expression of FOXO1 mRNA compared with the negative control group (Figure 3b). Similarly, western blot analysis indicated that the upregulation of miR-221-3p (miR-221-3p

mimics group) decreased the protein expression level of FOXO1. Moreover, downregulation of miR-221-3p (miR-221-3p inhibitor group) increased the protein expression level of FOXO1 (Figure 3c,d). These results indicated that miR-221-3p regulated FOXO1 expression.

2.5 | FOXO1 is highly expressed in DOR patients of advanced age

The FOXO1 expression level was quantified with qRT-PCR and western blot assay in human GCs isolated from aspirated follicular fluid. Results showed that the expression of FOXO1 in GCs from DOR patients of advanced age was significantly higher compared with its expression in young NOR individuals at both mRNA (Figure 4a) and protein (Figure 4b,c) levels.

FIGURE 3 The expression of miR-221-3p and *FOXO1* after transfection. qRT-PCR analysis of miR-221-3p expression (a), *FOXO1* expression (b) in KGN cells transfected with miRNA NC, miR-221-3p mimics, and miR-221-3p inhibitor. Western blot analysis of protein levels (c) and relative protein levels (d) of *FOXO1* in KGN cells transfected with miRNA NC, miR-221-3p mimics, and miR-221-3p inhibitor. *FOXO1*, Forkhead box transcription factor O1. * $p < .05$. miRNA, microRNA; qRT-PCR, quantitative reverse-transcription polymerase chain reaction

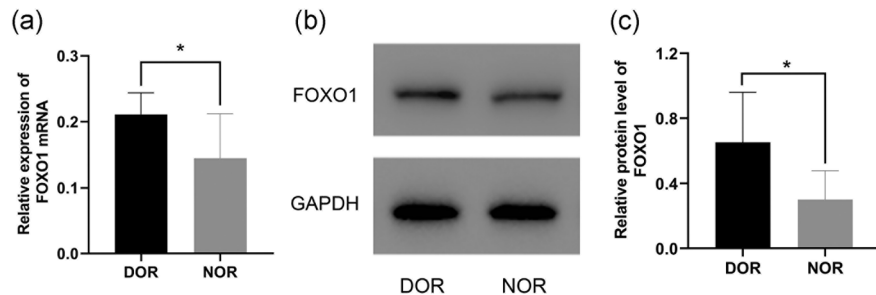
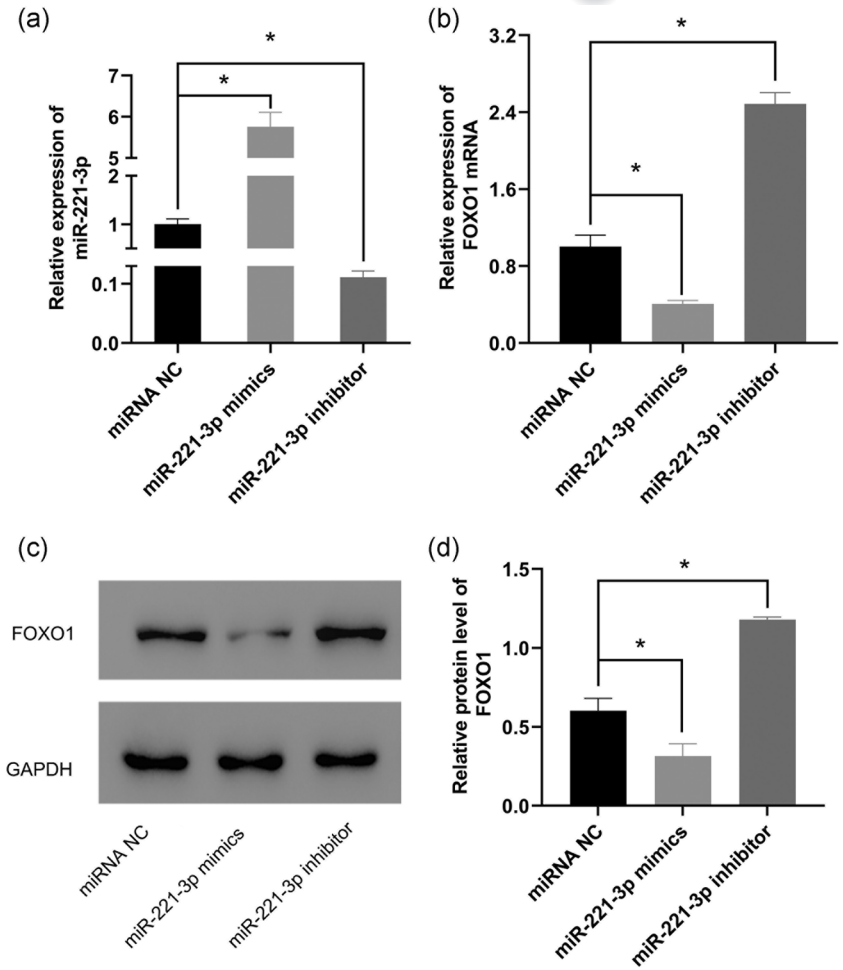


FIGURE 4 *FOXO1* expression was upregulated in human GCs from DOR patients. Relative *FOXO1* mRNA expression as determined by qRT-PCR in GCs from DOR and NOR group (a). Representative bands of *FOXO1* proteins as determined by western blot assay in GCs from DOR and NOR group (b). Quantification of *FOXO1* protein expression after normalization with GAPDH (c). GCs, granulosa cells. DOR, diminished ovarian reserve; *FOXO1*, Forkhead box transcription factor O1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger RNA; NOR, normal ovarian reserve; qRT-PCR, quantitative real-time polymerase chain reaction. * $p < .05$

3 | DISCUSSION

Aberrant GCs apoptosis has been associated with the pathogenesis of DOR. In our previous study, we reported that miR-221-3p was significantly downregulated in DOR patients of advanced age. However, the relationship between miR-221-3p and DOR has not been elucidated. In the present study, *FOXO1* was found to be a direct target gene of miR-221-3p based on results of integrated

bioinformatics prediction and dual-luciferase reporter assay. Besides, *BCL2L11*, the potential target gene was excluded by Dual-luciferase assay. Analysis of clinical GCs samples also revealed that mRNA and protein expression levels of *FOXO1* were significantly higher in DOR group compared with NOR group. Flow cytometry experiments further clarified that miR-221-3p regulates the apoptosis of GCs via targeting *FOXO1*, hence playing an important role in DOR.

A significant number of miRNAs are reported to be related to ovarian GCs apoptosis. But these studies have primarily focused on polycystic ovarian syndrome (PCOS). Besides, numerous studies have reported on the role of different miRNAs in GCs apoptosis in PCOS. Some miRNAs inhibit apoptosis, such as miR-129 (Zhu et al., 2020), miR-17-5p (G. Liu et al., 2020), miR-33b, and miR-142 (Y. Li et al., 2019), whereas others promote apoptosis, including miR-423 (Y. Li et al., 2019), miR-30d-5p (Yu & Liu, 2020), miR-135a (Wei et al., 2020), and miR-204 (X. Sun et al., 2019). It has been reported that miRNAs are differentially expressed in other ovarian disorders. For instance, miR-22-3p was significantly downregulated in premature ovarian failure (POF) patients and associated with apoptosis (Dang et al., 2015). miR-106a was downregulated in serum and GCs of women with DOR and was found to reduce GCs viability and promote apoptosis via enhancing apoptosis signal-regulating kinase 1 (ASK1) signaling (Hong et al., 2018). These studies illustrate that different miRNAs with differential expression pattern perform different functions in GCs apoptosis, thus playing different roles in follicular development. A previous study reported aberrant miRNAs expression profiles in GCs of young women with DOR (Woo et al., 2018). In our previous study (D. Liu et al., 2020), we compared miRNA expression profiles between DOR patients of advanced age and young women with NOR. Seventy miRNAs were found to be differentially expressed and among them, miR-221-3p was significantly downregulated in older DOR patients.

Previous studies on miR-221 have mainly focused on multiple cancers. miR-221 are reported to play a cancer-promoting role in breast cancer (Ye et al., 2016; Zong et al., 2019), gastric cancer (Zhou et al., 2019), osteosarcoma (OS) (Hu et al., 2019), ovarian cancer (J. Li et al., 2017; Xie et al., 2018), and bladder cancer (H. Liu et al., 2017), while it plays a tumor-suppressing role in laryngeal carcinoma (Shi et al., 2020), non-small cell lung cancer (Y. J. Sun et al., 2019), and pancreatic cancer (F. Li et al., 2018; J. Xie et al., 2018; Yang et al., 2018). Recent research on cattle found that miR-221 inhibited the production of E_2 and P_4 from GCs (Robinson et al., 2018). E_2 downregulated the expression of proapoptotic proteins ($p53$, Bax), and protected cells against oxidative (H_2O_2)-induced apoptosis (Almeida et al., 2018). However, there are relatively few studies on the effect of miR-221-3p on DOR and GCs apoptosis. One study reported that nine miRNAs, including miR-221, were found to be considerably lower in older individuals compared with young individuals. The study also revealed that miR-221 may be a potential marker of age-related diseases (Noren Hooten et al., 2010). In the present study, we investigated the mechanism of how miR-221-3p participates in DOR pathogenesis. We found that miR-221-3p modulated GCs apoptosis by targeting *FOXO1*.

Forkhead box transcription factor O1 (*FOXO1*) gene is a representative member of the *FOXO* family, localized on chromosome 13. *FOXO1* has been implicated in a variety of cellular processes, such as apoptosis, proliferation, oxidative stress, and inflammation (Kandula et al., 2016). In recent years, *FOXO1* has also been associated with tumor development and metabolic diseases, including obesity, diabetes, and atherosclerosis (Peng et al., 2020).

The activation of *FOXO1* is considered an attractive therapy towards cancers due to its tumor inhibition characteristics (Link, 2019). Previously, *FOXO1* was reported to regulate apoptosis of mouse granulosa cells (Shen et al., 2014). Mechanistically, it targets several downstream target genes, such as *FasI*, *TRAIL*, and *PINK* (Link, 2019). Thus, silencing *FOXO1* may be a potential treatment strategy for some diseases associated with excessive apoptosis, such as DOR. miRNAs have been confirmed to serve a substantial role in disrupting or weakening *FOXO1* translation. Besides, *FOXO1* has been shown to be negatively regulated by many miRNAs, including miR-182 (Huang et al., 2018), miR-183-5p (Shang et al., 2020), miR-9 (Aishanjiang et al., 2018), and miR-27a (Zhang et al., 2019). In the present study, miR-221-3p which is at low levels in DOR patients, was inversely related to *FOXO1*.

The disorder of GCs' apoptosis could influence the connection between GCs themselves and between GCs and the oocyte. Therefore, altering the GCs' apoptotic state can significantly influence oocyte's development. In a previous study, ovarian granulosa-like cells (OGLCs), induced differentiation from human induced pluripotent stem cells (iPSCs), were grafted to POF murine model. It was found that OGLCs grow efficiently thereby promoting the formation of mature follicles and decreasing formation of atretic follicles (T. Liu et al., 2016). This indicates that inhibiting the apoptosis of GCs can significantly improve oocyte competence. In an ovarian oxidative stress model, low expression of miR-145 promoted GCs apoptosis by upregulating the expression of krüppel-like factor 4 (*KLF4*; Xu et al., 2017). miR-145 protects GCs from apoptosis induced by oxidative stress by suppressing the expression of *KLF4*. Another study reported that increased expression of miR-21 in mesenchymal stem cells (MSCs) improved the efficacy of chemotherapy-induced POF, specifically by increasing ovarian weight, the number of follicles, E_2 levels and decreasing FSH level. This repair capacity was associated with the fact that miR-21 suppressed GCs apoptosis via targeting phosphatase and tensin homolog deleted on chromosome ten and programmed cell death 4 (Fu et al., 2017). These findings provide insights into other possible avenues to be explored for the treatment of DOR patients by developing miR-221-3p analogs or short peptides specifically to inhibit *FOXO1* activity.

Even minute levels of miRNA can regulate the expression of multiple genes simultaneously. This implies that a minor change in the miRNA pattern could have profound effects on cell function. Therefore, miR-based treatments can be regarded as promising therapeutic strategies compared with conventional single-target therapies (Shah et al., 2016).

In the present study, a possible new mechanism of DOR pathogenesis which might guide the development of future potential treatment for DOR patients suffering from infertility was found. However, there were limitations to this study. Only in vitro experiments were performed. In vivo animal experiments are needed to further confirm these findings.

In conclusion, this study demonstrates that the downregulation of miR-221-3p may upregulate *FOXO1* expression and hence contribute to the pathogenesis of DOR by promoting apoptosis of GCs.

Moreover, we have shown that the low miR-221-3p expression and the high *FOXO1* expression in DOR patients can be considered as potential diagnostic markers and therapeutic targets of DOR.

4 | MATERIALS AND METHODS

4.1 | Research subjects

A total of forty patients undergoing assisted reproductive technology treatment in a Reproductive and Genetic Center in the Affiliated Hospital of Shandong University of Traditional Chinese Medicine were enrolled in this study from January 2019 to December 2019. Among them were 20 older women (aged than 35 years), diagnosed with DOR and 20 young women with NOR suffering from infertility caused by tubal factors. All participants signed written informed consent. Research ethics approval was obtained from the Reproductive Medicine Ethics Committee of the Affiliated Hospital of the Shandong University of Traditional Chinese Medicine. The study was registered with the Chinese Clinical Trial Register (Registration number: ChiCTR1800019798). DOR was diagnosed in line with the revised version of Bologna diagnostic criteria. Participants who met at least two of the following three items (Ferraretti et al., 2011) were enrolled: (i) Bilateral AFC less than 6, (ii) AMH less than 2 ng/ml, (iii) basal FSH on the third day of menstruation of 10–40 mIU/mL.

All patients were subjected to the antagonist protocol for ovulation induction. On the third day of menstruation, serum FSH, LH, E₂, P₄, and vaginal ultrasound were examined to evaluate the basic status of the ovary. If the basic conditions were permissive, then, gonadotropin (Gn) was used to initiate controlled ovarian hyperstimulation (COH). Gn included human recombinant follicle-stimulating hormone (rFSH, GonalF), human menopausal gonadotropin (HMG), and human recombinant luteinizing hormone (rLH, Luveris, Merck Serono). The choice of Gn dosage and type was decided according to the patient's characteristics (age, BMI, and AFC). A GnRH antagonist (Cetrotide 0.25 mg, Merck Serono) was administered when the leading follicle reached a diameter of 12–14 mm until the trigger day. When at least two follicles more than or equal to 18 mm or three follicles more than or equal to 17 mm in diameter were formed, 4000–10,000 IU of human chorionic gonadotropin (hCG) or 250 µg recombinant human chorionic gonadotrophin (rhCG, Ovidrel™) was administered to trigger ovulation. The dosage of these hormones was determined based on the number of follicles and the blood hormone levels. Oocytes pick-up was performed by ultrasound-guided transvaginal oocyte retrieval, at 36 h after ovulation triggering, and the follicular fluid was also collected.

4.2 | Extraction of granulosa cells

The follicular fluid was centrifuged at 2000 rpm for 5 min and the supernatant was removed by aspiration. To the precipitate, 3-ml phosphate buffer saline (PBS) was added and mixed. About 5 ml of the lymphocyte separation solution (TBD) was added to a 15-ml

centrifuge tube. Subsequently, the precipitate was slowly dropped to the upper layer of the lymphocyte separation solution and centrifuged at 1500 rpm for 20 min. The GCs in the layer between the suspension and lymphocyte separation solution were aspirated and transferred to a 15-ml centrifuge tube containing 5-ml PBS, mixed well, and centrifuged at 2000 rpm for 5 min. The supernatant was discarded and the GCs were transferred to a 1.5-ml centrifuge tube and centrifuged at 1500 rpm for 2 min. The GCs were preserved in a –80°C refrigerator for further analysis.

4.3 | Cell culture

HEK-293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; hyclone) supplemented with 10% fetal bovine serum (FBS, Gibco). KGN cells (a human granulosa carcinoma cell line) were cultured in special medium (Procell, CM-0603) containing 10% FBS. All the cells were incubated at 37°C with 5% CO₂.

4.4 | miR-221-3p target gene prediction

Target genes for miR-221-3p were predicted using TargetScan (<http://www.targetscan.org>) and DINAN (<http://diana.imis.athena-innovation.gr/DianaTools/>) software. The intersections of target genes were selected and target genes associated with apoptosis, *FOXO1*, and *BCL2L11*, were identified and verified.

4.5 | Dual-luciferase reporter experiments

miR-221-3p mimics, miRNA negative control (NC), pmirGLO-*FOXO1*-wild type(WT)/mutation (MT; Figure 1a), and pmirGLO-*BCL2L1*-WT/MT vectors (Figure 1b) were synthesized by JINKAIRUI Bioengineering Co., Ltd. 293T cells were seeded into 24-well culture plates, and transfected with the corresponding vectors when the cells attained 70%–80% confluence. After dissolution in DEPC water, 2 µl miRNA, and 2 µg plasmid were added to a 50 µl serum-free DMEM medium and incubated at room temperature for 5 min. Various combinations of plasmids are shown in Table 1. Besides, 2 µl Lipofectamine 2000 (Invitrogen) was added to a 50 µl serum-free DMEM medium, mixed and incubated at room temperature for 15 min. The original culture medium was aspirated, and 100 µl of the previous mixture was added to 1 well of a 24-well plate, and then filled up to 500 µl with serum-free DMEM culture medium. After incubation at 37°C for 4–6 h, the culture media was removed and replaced with a 0.5 ml complete medium and incubated at 37°C for 48 h. The cell-culture medium was aspirated and 200 µl of cell lysate (RG027-1) was added and incubated at room temperature for 10 min. The cells were lysed and the cell lysate was collected and centrifuged at 10,000 rpm for 5 min. The supernatants were stored until further use. The Firefly and renilla luciferase activities were measured using the Dual-Luciferase Reporter Gene Assay Kit

(Biovision) according to the manufacturer's instructions. Relative luciferase activity was calculated as follows: Firefly luciferase/Renilla luciferase.

4.6 | Cell transfection

KGN cells were seeded into 6-well plates at a density of 3×10^5 cells per well. Transfection of miR-221-3p mimics, miR-221-3p inhibitor, or miRNA NC was performed using Lipofectamine 2000 (Invitrogen) when cells reached 60% confluence according to the manufacturer's protocol. After 48 h of transfection, KGN cells were harvested for subsequent analysis (AnnexinV-fluorescein isothiocyanate/propidium iodide [FITC/PI] cell apoptosis detection, western blot, and quantitative real-time polymerase chain reaction).

4.7 | Detection of cell apoptosis via flow cytometry

Cell apoptosis was evaluated by flow cytometry using the Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime). Briefly, cells transfected with miR-221-3p mimics, miR-221-3p inhibitor, or miRNA NC, were digested with 0.25% trypsin. After washing with PBS, cell suspension was prepared. About 5 μ l Annexin V-FITC was added to the cell suspension and incubated at 4°C in darkness for 15 min after which and 5 μ l propidium iodide (PI) was added and incubated at 4°C for 5 min. Cell apoptosis was analyzed by flow cytometry using Flowjo software.

4.8 | Quantitative real-time polymerase chain reaction

The primer sequences shown in Table 2 for miR-221-3p, FOXO1, U6, and GAPDH were synthesized by JRDUN Biotechnology Co. Total RNA was isolated from clinical GCs samples and KGN cells (transfected with miR-221-3p mimics, inhibitor, or NC) using Trizol[®] Reagent (Invitrogen) according to the manufacturer's instructions. The cDNAs were synthesized using ReverTra Ace[®] qPCR RT kit (Toyobo). Quantitative PCR was carried out using SYBR Green qPCR Master Mix according to the manufacturer's instructions. Relative gene expression was normalized to the expression of internal reference gene (GAPDH for FOXO1 and U6 for miR-221-3p). All the reactions were performed in triplicates and repeated three times. Relative gene expression was calculated using $2^{-\Delta\Delta C_t}$ method for clinical GCs samples and $2^{-\Delta\Delta C_t}$ method for KGN cells (Schmittgen & Livak, 2008).

4.9 | Western blot

The total proteins were extracted from clinical GCs samples and KGN cells (transfected with miR-221-3p mimics, inhibitor, or NC) using radioimmunoprecipitation assay lysis (Boster) according to the

manufacturer's instructions. The protein concentration was determined using a Bicinchoninic Acid Kit (Boster). After adjusting for the protein concentration of each sample equally, five times sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added and boiled for 5 min. Next, equal amount of proteins (50 μ g) were electrophoresed by SDS-PAGE and subsequently transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skimmed milk powder in TBST at room temperature for 1 h while shaking, followed by incubation with primary antibody against FOXO1 (1:1000; #2880, CST) and GAPDH (1:2000; #5174, CST) overnight at 4°C, and horseradish peroxidase secondary antibody (1:1000, goat anti-rabbit, A0208, Beyotime) at room temperature for 1.5 h. Finally, the proteins were visualized with an electrochemiluminescence solution and analyzed using the ImageJ software.

4.10 | Statistical analysis

Statistical analysis was performed with SPSS version 21.0. Data are presented as mean \pm standard deviation (SD). A student's t-test was performed to compare data between two groups. One-way analysis of variance was performed to compare data among multiple groups. $p < .05$ was considered statistically significant.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The datasets generated and analyzed during the current study are available from the corresponding author upon request.

REFERENCES

- Aishanjiang, A., Rouzi, N., Jiao, Z., Wang, L., Wusainahong, K., Wumanjiang, N., & Niyazi, M. (2018). MicroRNA-9 enhances invasion and migration of cervical carcinomas by directly targeting FOXO1. *European Review for Medical and Pharmacological Sciences*, 22(8), 2253–2260.
- Almeida, C. P., Ferreira, M. C. F., Silveira, C. O., Campos, J. R., Borges, I. T., Baeta, P. G., Silva, F. H. S., Reis, F. M., & Del Puerto, H. L. (2018). Clinical correlation of apoptosis in human granulosa cells-A review. *Cell Biology International*, 42(10), 1276–1281. <https://doi.org/10.1002/cbin.11036>
- Andreas, E., Hoelker, M., Neuhooff, C., Tholen, E., Schellander, K., Tesfaye, D., & Salilew-Wondim, D. (2016). MicroRNA 17-92 cluster regulates proliferation and differentiation of bovine granulosa cells

- by targeting PTEN and BMP2 genes. *Cell and Tissue Research*, 366(1), 219–230. <https://doi.org/10.1007/s00441-016-2425-7>
- Dang, Y., Zhao, S., Qin, Y., Han, T., Li, W., & Chen, Z. J. (2015). MicroRNA-22-3p is down-regulated in the plasma of Han Chinese patients with premature ovarian failure. *Fertility and Sterility*, 103(3), 802–807. <https://doi.org/10.1016/j.fertnstert.2014.12.106>
- Devine, K., Mumford, S. L., Wu, M., DeCherney, A. H., Hill, M. J., & Propst, A. (2015). Diminished ovarian reserve in the United States assisted reproductive technology population: Diagnostic trends among 181,536 cycles from the Society for Assisted Reproductive Technology Clinic Outcomes Reporting System. *Fertility and Sterility*, 104(3), 612–619. <https://doi.org/10.1016/j.fertnstert.2015.05.017>
- Ferraretti, A. P., La Marca, A., Fauser, B. C., Tarlatzis, B., Nargund, G., & Gianaroli, L. (2011). ESHRE consensus on the definition of 'poor response' to ovarian stimulation for in vitro fertilization: The Bologna criteria. *Human Reproduction*, 26(7), 1616–1624. <https://doi.org/10.1093/humrep/der092>
- Fu, X., He, Y., Wang, X., Peng, D., Chen, X., Li, X., & Wang, Q. (2017). Overexpression of miR-21 in stem cells improves ovarian structure and function in rats with chemotherapy-induced ovarian damage by targeting PDCD4 and PTEN to inhibit granulosa cell apoptosis. *Stem Cell Research & Therapy*, 8(1), 187. <https://doi.org/10.1186/s13287-017-0641-z>
- Hong, L., Peng, S., Li, Y., Fang, Y., Wang, Q., Klausen, C., Yin, C., Wang, S., Leung, P. C. K., & Yang, X. (2018). miR-106a increases granulosa cell viability and is downregulated in women with diminished ovarian reserve. *Journal of Clinical Endocrinology and Metabolism*, 103(6), 2157–2166. <https://doi.org/10.1210/jc.2017-02344>
- Hu, X. H., Zhao, Z. X., Dai, J., Geng, D. C., & Xu, Y. Z. (2019). MicroRNA-221 regulates osteosarcoma cell proliferation, apoptosis, migration, and invasion by targeting CDKN1B/p27. *Journal of Cellular Biochemistry*, 120(3), 4665–4674. <https://doi.org/10.1002/jcb.27755>
- Huang, Y., Fan, X., Tao, R., Song, Q., Wang, L., Zhang, H., Kong, H., & Huang, J. (2018). Effect of miR-182 on hepatic fibrosis induced by *Schistosomiasis japonica* by targeting FOXO1 through PI3K/AKT signaling pathway. *Journal of Cellular Physiology*, 233(10), 6693–6704. <https://doi.org/10.1002/jcp.26469>
- Kandula, V., Kosuru, R., Li, H., Yan, D., Zhu, Q., Lian, Q., Ge, R., Xia, Z., & Irwin, M. G. (2016). Forkhead box transcription factor 1: Role in the pathogenesis of diabetic cardiomyopathy. *Cardiovascular Diabetology*, 15, 44. <https://doi.org/10.1186/s12933-016-0361-1>
- Kittlmann, S., & McGregor, A. P. (2019). Modulation and evolution of animal development through microRNA regulation of gene expression. *Genes*, 10(4), 321. <https://doi.org/10.3390/genes10040321>
- Komatsu, K., & Masubuchi, S. (2018). Mouse oocytes connect with granulosa cells by fusing with cell membranes and form a large complex during follicle development. *Biology of Reproduction*, 99(3), 527–535. <https://doi.org/10.1093/biolre/i0y072>
- Li, F., Xu, J. W., Wang, L., Liu, H., Yan, Y., & Hu, S. Y. (2018). MicroRNA-221-3p is up-regulated and serves as a potential biomarker in pancreatic cancer. *Artificial Cells, Blood Substitutes, and Biotechnology*, 46(3), 482–487. <https://doi.org/10.1080/21691401.2017.1315429>
- Li, J., Li, Q., Huang, H., Li, Y., Li, L., Hou, W., & You, Z. (2017). Overexpression of miRNA-221 promotes cell proliferation by targeting the apoptotic protease activating factor-1 and indicates a poor prognosis in ovarian cancer. *International Journal of Oncology*, 50(4), 1087–1096. <https://doi.org/10.3892/ijo.2017.3898>
- Li, Y., Xiang, Y., Song, Y., Wan, L., Yu, G., & Tan, L. (2019). Dysregulated miR-142, -33b and -423 in granulosa cells target TGFBR1 and SMAD7: A possible role in polycystic ovary syndrome. *Molecular Human Reproduction*, 25(10), 638–646. <https://doi.org/10.1093/molehr/gaz014>
- Link, W. (2019). Introduction to FOXO Biology. *Methods in Molecular Biology*, 1890, 1–9. https://doi.org/10.1007/978-1-4939-8900-3_1
- Liu, D., Lian, F., Xiang, S., Guo, Y., Wu, H., & Qiu, Y. (2020). MicroRNAs with altered expression profiles in granulosa of women of advanced age with diminished ovarian reserve. *Journal of Medical Imaging and Health Informatics*, 10(10), 2316–2323. <https://doi.org/10.1166/jmih.2020.2988>
- Liu, G., Liu, S., Xing, G., & Wang, F. (2020). lncRNA PVT1/microRNA-17-5p/PTEN axis regulates secretion of E2 and P4, proliferation, and apoptosis of ovarian granulosa cells in PCOS. *Molecular Therapy: Nucleic Acids*, 20, 205–216. <https://doi.org/10.1016/j.omtn.2020.02.007>
- Liu, H., Chang, J. K., Hou, J. Q., Zhao, Z. H., & Zhang, L. D. (2017). Inhibition of miR-221 influences bladder cancer cell proliferation and apoptosis. *European Review for Medical and Pharmacological Sciences*, 21(14), 3193–3199.
- Liu, J., Li, X., Yao, Y., Li, Q., Pan, Z., & Li, Q. (2018). miR-1275 controls granulosa cell apoptosis and estradiol synthesis by impairing LRH-1/CYP19A1 axis. *Biochimica et Biophysica Acta, Gene Regulatory Mechanisms*, 1861(3), 246–257. <https://doi.org/10.1016/j.bbagr.2018.01.009>
- Liu, T., Li, Q., Wang, S., Chen, C., & Zheng, J. (2016). Transplantation of ovarian granulosa-like cells derived from human induced pluripotent stem cells for the treatment of murine premature ovarian failure. *Molecular Medicine Reports*, 13(6), 5053–5058. <https://doi.org/10.3892/mmr.2016.5191>
- Murase, T., Iwase, A., Komatsu, K., Bayasula, Nakamura, T., Osuka, S., Takikawa, S., Goto, M., Kotani, T., & Kikkawa, F. (2018). Follicle dynamics: Visualization and analysis of follicle growth and maturation using murine ovarian tissue culture. *Journal Of Assisted Reproduction And Genetics*, 35(2), 339–343. <https://doi.org/10.1007/s10815-017-1073-5>
- Noren Hooten, N., Abdelmohsen, K., Gorospe, M., Ejiogu, N., Zonderman, A. B., & Evans, M. K. (2010). microRNA expression patterns reveal differential expression of target genes with age. *PLoS One*, 5(5), e10724. <https://doi.org/10.1371/journal.pone.0010724>
- Peng, S., Li, W., Hou, N., & Huang, N. (2020). A Review of FoxO1-regulated metabolic diseases and related drug discoveries. *Cells*, 9(1), 184. <https://doi.org/10.3390/cells9010184>
- Robinson, C. L., Zhang, L., Schütz, L. F., Totty, M. L., & Spicer, L. J. (2018). MicroRNA 221 expression in theca and granulosa cells: Hormonal regulation and function. *Journal of Animal Science*, 96(2), 641–652. <https://doi.org/10.1093/jas/skx069>
- Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nature Protocols*, 3(6), 1101–1108. <https://doi.org/10.1038/nprot.2008.73>
- Shah, M. Y., Ferrajoli, A., Sood, A. K., Lopez-Berestein, G., & Calin, G. A. (2016). microRNA Therapeutics in Cancer: An emerging concept. *EBioMedicine*, 12, 34–42. <https://doi.org/10.1016/j.ebiom.2016.09.017>
- Shang, A., Wang, X., Gu, C., Liu, W., Sun, J., Zeng, B., Chen, C., Ji, P., Wu, J., Quan, W., Yao, Y., Wang, W., Sun, Z., & Li, D. (2020). Exosomal miR-183-5p promotes angiogenesis in colorectal cancer by regulation of FOXO1. *Aging (Albany NY)*, 12(9), 8352–8371. <https://doi.org/10.18632/aging.103145>
- Shen, M., Liu, Z., Li, B., Teng, Y., Zhang, J., Tang, Y., Sun, S. C., & Liu, H. (2014). Involvement of FoxO1 in the effects of follicle-stimulating hormone on inhibition of apoptosis in mouse granulosa cells. *Cell Death & Disease*, 5(10), e1475. <https://doi.org/10.1038/cddis.2014.400>
- Shi, C., Wu, Y. Y., & Wei, L. Q. (2020). MiR-221 affects the proliferation and apoptosis of laryngeal cancer cells through the PI3K/AKT signaling pathway. *European Review for Medical and Pharmacological Sciences*, 24(3), 1258–1263.
- Sun, X., Su, S., Zhang, G., Zhang, H., & Yu, X. (2019). MiR-204 suppresses cell proliferation and promotes apoptosis in ovarian granulosa cells

- via targeting TPT1 in polycystic ovary syndrome. *Biochemistry and Cell Biology*, 97(5), 554–562. <https://doi.org/10.1139/bcb-2019-0019>
- Sun, Y. J., Li, J., & Chen, C. H. (2019). Effects of miR-221 on the apoptosis of non-small cell lung cancer cells by lncRNA HOTAIR. *European Review for Medical and Pharmacological Sciences*, 23(10), 4226–4233. https://doi.org/10.26355/eurrev_201905_17927
- Tang, J., Xu, J., Zhi, Z., Wang, X., Wang, Y., Zhou, Y., & Chen, R. (2019). MiR-876-3p targets KIF20A to block JAK2/STAT3 pathway in glioma. *American Journal of Translational Research*, 11(8), 4957–4966.
- Wang, M., Liu, M., Sun, J., Jia, L., Ma, S., Gao, J., Xu, Y., Zhang, H., Tsang, S. Y., & Li, X. (2017). MicroRNA-27a-3p affects estradiol and androgen imbalance by targeting Creb1 in the granulosa cells in mouse polycystic ovary syndrome model. *Reproductive biology*, 17(4), 295–304. <https://doi.org/10.1016/j.repbio.2017.09.005>
- Wang, Y., Li, Y., Chen, R., Cui, X., Yu, J., & Liu, Z. (2016). Electroacupuncture for reproductive hormone levels in patients with diminished ovarian reserve: A prospective observational study. *Acupuncture in Medicine*, 34(5), 386–391. <https://doi.org/10.1136/acupmed-2015-011014>
- Wei, Y., Lu, S., Hu, Y., Guo, L., Wu, X., Liu, X., & Sun, Y. (2020). MicroRNA-135a regulates VEGFC expression and promotes luteinized granulosa cell apoptosis in polycystic ovary syndrome. *Reproductive Sciences*, 27(7), 1436–1442. <https://doi.org/10.1007/s43032-020-00155-0>
- Woo, I., Christenson, L. K., Gunewardena, S., Ingles, S. A., Thomas, S., Ahmady, A., Chung, K., Bendikson, K., Paulson, R., & McGinnis, L. K. (2018). Micro-RNAs involved in cellular proliferation have altered expression profiles in granulosa of young women with diminished ovarian reserve. *Journal of Assisted Reproduction And Genetics*, 35(10), 1777–1786. <https://doi.org/10.1007/s10815-018-1239-9>
- Xie, J., Wen, J. T., Xue, X. J., Zhang, K. P., Wang, X. Z., & Cheng, H. H. (2018). MiR-221 inhibits proliferation of pancreatic cancer cells via down regulation of SOCS3. *European Review for Medical and Pharmacological Sciences*, 22(7), 1914–1921. https://doi.org/10.26355/eurrev_201804_14714
- Xie, X., Huang, Y., Chen, L., & Wang, J. (2018). miR-221 regulates proliferation and apoptosis of ovarian cancer cells by targeting BMF. *Oncology Letters*, 16(5), 6697–6704. <https://doi.org/10.3892/ol.2018.9446>
- Xu, L., Sun, H., Zhang, M., Jiang, Y., Zhang, C., Zhou, J., Ding, L., Hu, Y., & Yan, G. (2017). MicroRNA-145 protects follicular granulosa cells against oxidative stress-induced apoptosis by targeting Krüppel-like factor 4. *Molecular and Cellular Endocrinology*, 452, 138–147. <https://doi.org/10.1016/j.mce.2017.05.030>
- Yang, Y., Sun, Y., Wang, H., Li, H., Zhang, M., Zhou, L., Meng, X., Wu, Y., Liu, P., Liu, X., Zhang, J., & Tan, X. (2018). MicroRNA-221 induces autophagy through suppressing HDAC6 expression and promoting apoptosis in pancreatic cancer. *Oncology Letters*, 16(6), 7295–7301. <https://doi.org/10.3892/ol.2018.9513>
- Yao, L., Li, M., Hu, J., Wang, W., & Gao, M. (2018). MiRNA-335-5p negatively regulates granulosa cell proliferation via SGK3 in PCOS. *Reproduction*, 156(5), 439–449. <https://doi.org/10.1530/rep-18-0229>
- Ye, Z., Hao, R., Cai, Y., Wang, X., & Huang, G. (2016). Knockdown of miR-221 promotes the cisplatin-inducing apoptosis by targeting the BIM-Bax/Bak axis in breast cancer. *Tumour Biology*, 37(4), 4509–4515. <https://doi.org/10.1007/s13277-015-4267-4>
- Yu, M., & Liu, J. (2020). MicroRNA-30d-5p promotes ovarian granulosa cell apoptosis by targeting Smad2. *Experimental and Therapeutic Medicine*, 19(1), 53–60. <https://doi.org/10.3892/etm.2019.8184>
- Zhang, L. Y., Chen, Y., Jia, J., Zhu, X., He, Y., & Wu, L. M. (2019). MiR-27a promotes EMT in ovarian cancer through active Wnt/ β -catenin signalling by targeting FOXO1. *Cancer Biomarkers: Section A of Disease Markers*, 24(1), 31–42. <https://doi.org/10.3233/cbm-181229>
- Zhou, Q. Y., Peng, P. L., & Xu, Y. H. (2019). MiR-221 affects proliferation and apoptosis of gastric cancer cells through targeting SOCS3. *European Review for Medical and Pharmacological Sciences*, 23(21), 9427–9435. https://doi.org/10.26355/eurrev_201911_19436
- Zhu, H. L., Chen, Y. Q., & Zhang, Z. F. (2020). Downregulation of lncRNA ZFAS1 and upregulation of microRNA-129 repress endocrine disturbance, increase proliferation and inhibit apoptosis of ovarian granulosa cells in polycystic ovarian syndrome by downregulating HMGB1. *Genomics*, 112(5), 3597–3608. <https://doi.org/10.1016/j.ygeno.2020.04.011>
- Zong, Y., Zhang, Y., Sun, X., Xu, T., Cheng, X., & Qin, Y. (2019). miR-221/222 promote tumor growth and suppress apoptosis by targeting lncRNA GAS5 in breast cancer. *Bioscience Reports*, 39(1), <https://doi.org/10.1042/bsr20181859>

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