Abstract. The present study aimed to investigate whether microRNA (miR)-451a plays a role in polycystic ovary syndrome by regulating the biological function of ovarian granulosa cells and investigate the underlying molecular mechanism. In the present study, reverse transcription-quantitative PCR (RT-qPCR) analysis detected markedly low expression of miR-451a in KGN cells. TargetScan predicted that cyclic AMP-dependent transcription factor ATF-2 (ATF2) was a potential target gene of miR-451a, which was confirmed by a Dual-Luciferase reporter gene assay. Moreover, western blotting and RT-qPCR experiments indicated that ATF2 was significantly overexpressed in KGN cells. In addition, western blotting and RT-qPCR experiments were utilized to assess cell transfection efficiency, and it was found that miR-451a mimic significantly increased miR-451a expression in KGN cells. Subsequently, MTT assay was performed to detect cell proliferation and flow cytometry was utilized to detect cell apoptosis. Western blot and RT-qPCR assays were utilized to assess the protein and mRNA expression of ATF2 and cyclin D1. The results confirmed that miR-451a mimic significantly decreased ATF2 protein and mRNA expression in KGN cells, and this decrease was reversed by ATF2-plasmid co-transfection. Moreover, miR-451a mimic inhibited cell proliferation, enhanced cell apoptosis, reduced cyclin D1 expression, increased caspase-3 activity and cleaved caspase-3 protein levels, while it reduced pro-caspase 3 protein levels in KGN cells, and these effects were significantly reversed by ATF2-plasmid. The present preliminary results demonstrated that miR-451a regulated the proliferation and apoptosis of ovarian granulosa cells by targeting ATF2. Thus, the miR-451a/ATF2 axis may be a new potential target for the treatment of polycystic ovary syndrome.

Introduction

Polycystic ovary syndrome (PCOS) is an endocrine disorder that causes health problems in 6-10% of premenopausal women (1). Hyperandrogenemia with chronic anovulation is the main phenotype of PCOS (2). The typical clinical manifestations of PCOS include acanthosis, alopecia, subfertility, acne vulgaris, obesity, seborrhea (3), hirsutism and menstrual disorders (4). PCOS is one of the main causes of anovulatory sterility in women (5). Furthermore, women with PCOS are at an increased risk of dyslipidemia, insulin resistance, type 2 diabetes and hypertension (6). However, although current research has provided more information on genetic background and the effect of environmental factors, the mechanism underlying the development of PCOS remains elusive.

During the development of ovarian follicles, it was observed that all stages of follicular atresia are associated with the apoptosis of granulosa cells (GCs). Based on this observation, GC apoptosis is considered to be the main mechanism underlying follicular atresia. A variety of factors have been shown to cause GC apoptosis, including depletion of cell survival factors (7). In addition, there is evidence that abnormal GC function may be the cause of abnormal follicle formation in PCOS (8). The high rate of apoptotic GCs is associated with low pregnancy rates, fertilization rates and embryonic dysplasia (9). However, the exact etiology of PCOS remains to be fully elucidated.

MicroRNAs (miRs/miRNAs) are highly conserved single-stranded non-coding RNA molecules composed of 20-24 nucleotides. miRNAs are important regulators of biological processes, such as cell proliferation, differentiation, migration and apoptosis (10). Dysregulation and differential expression of miRNAs have been associated with ovarian cancer, PCOS and uterus-related diseases. Furthermore, miRNAs have been found to be crucial for reproductive function, gonadal development and sex differentiation (11). miR-451a is located on human chromosome 17q11.2. A previous study by Ibáñez et al suggested that one of the
biovarkers for the diagnosis and treatment of PCOS during puberty may be circulating miR-451a (12). Low circulating levels of miR-451a in women with PCOS were previously reported, but its specific role remained unclear (13).

Although the pathogenic mechanism of PCOS must be further elucidated, previous findings indicated that the proliferation and survival of GCs may be implicated. The ovarian granulosa cell tumour cell line KGN has been widely used to investigate the biological function of ovarian granulosa cells in PCOS (14-16). Therefore, the aim of the present study was to determine whether miR-451a could regulate the biological function of KGN cells to verify its role in PCOS and to further explore the underlying molecular mechanisms, in order to provide new insights into the treatment of PCOS.

**Materials and methods**

**Cell culture, treatment and transfection.** Human ovarian granulosa cell tumour cell line KGN cells, normal ovarian surface epithelial IOSE80 cells and 293 cells were all purchased from the American Type Culture Collection. All cell lines were cultured in DMEM/F12 supplemented with 10% FBS, 100 U/ml penicillin G (Life Technologies; Thermo Fisher Scientific, Inc.) and 0.1 mg/ml streptomycin sulfate (Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator at 37˚C with 5% CO₂.

For the cell transfection experiments, vectors including miR-451a mimic, miR-451a mimic control, control-plasmid, ATF2-plasmid, or miR-451a mimic + ATF2-plasmid were transfected into KGN cells using Lipofectamine™ 2000 (Life Technologies; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

**Target prediction and luciferase reporter assay.** TargetScan version 7.2 (www.targetscan.org) was used to analyze and predict potential targets for miR-451a. The 3'-untranslated region (3'UTR) sequence of mutated (MUT) or wild-type (WT) ATF2, containing the putative miR-451a-binding site, was amplified by PCR and cloned into a psiCHECK vector (Promega Corporation). Site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis kit (Promega Corporation). Site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis kit (Promega Corporation). Site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis kit (Promega Corporation). Site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis kit (Promega Corporation). Site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis kit (Promega Corporation). Site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis kit (Promega Corporation). Site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis kit (Promega Corporation). Site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis kit (Promega Corporation). Site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis kit (Promega Corporation). Site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis kit (Promega Corporation). Site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis kit (Promega Corporation). Site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis kit (Promega Corporation). Site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis kit (Promega Corporation). Site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis kit (Promega Corporation). Site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis kit (Promega Corporation). Site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis kit (Promega Corporation). Site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis kit (Promega Corporation). Site-directed mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis kit (Promega Corporation).

**Determination of cell proliferation.** MTT analysis was used to assess the proliferation of KGN cells. Briefly, KGN cells were seeded into 96-well plates at a density of 5x10³ cells/well. KGN cells were transfected with the vectors (mimic control, miR-451a mimic, miR-451a mimic + ATF2-plasmid or miR-451a mimic + control-plasmid) and incubated for 48 h. KGN cells were incubated with 20 µl MTT for 4 h at 37˚C. The cells were then lysed with 150 µl DMSO for 10 min at room temperature at 24, 48, 72 and 96 h after transfection.

**Flow cytometric analysis of apoptosis.** KGN cells (5x10³ cells per well) transfected with indicated plasmids or oligonucleotides were seeded in 6-well plates and incubated for 48 h. For cell apoptosis analysis, the Annexin V-fluorescein isothiocyanate-propidium iodide Apoptosis Detection kit (Abcam) was used according to the manufacturer's instructions. Cell apoptosis was detected using the FACScan flow cytometry system equipped with CellQuest software version 5.1 (Becton, Dickinson and Company).

**Caspase-3 activity detection.** After transfection for 48 h, KGN cells were collected through centrifugation (600 x g; 4˚C;
5 min). Then the caspase-3 activity was immediately detected using a caspase-3 activity assay kit (cat no. C1116; Beyotime Institute of Biotechnology), following with the manufacturer’s protocol.

Western blotting. Total protein was extracted from cells using 1 ml RIPA cell lysis buffer (Sangon Biotech, Co., Ltd.). The proteins were quantified using a bicinchoninic acid protein assay reagent (Thermo Fisher Scientific, Inc.). A total of 40 µg protein from each sample was separated by 10% SDS-PAGE, and subsequently transferred onto PVDF membranes (Bio-Rad Laboratories, Inc.). The membranes were blocked for 1 h in TBS with 0.1% Tween-20 in 5% skimmed milk at room temperature, and incubated overnight at 4˚C with the following primary antibodies: Anti-ATF2 (cat no. 35031; 1:1,000; Cell Signaling Technology, Inc.), anti-cyclin D1 (cat no. 55506; 1:10,000; Cell Signaling Technology, Inc.), anti-cleaved caspase-3 (cat no. ab32042; 1:500; Abcam), anti-pro-caspase-3 (cat no. ab3499; 1:500; Abcam) and anti-GAPDH (cat no. 5174; 1:1,000, Cell Signaling Technology, Inc.). This was followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (cat no. 7074; 1:1,000; Cell Signaling Technology, Inc.) for 1 h at room temperature. Finally, enhanced chemiluminescence (EMD Millipore) was used to examine the immune complexes and the protein bands were quantified using ImageJ software (version 2.0; National Institutes of Health).

Statistical analysis. All experiments were performed three times. Data are expressed as the mean ± SD and evaluated using Student’s t-test or one-way ANOVA followed by Tukey’s post hoc test. Data were analyzed using SPSS 22.0 software (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-451a expression in the IOSE80 and KGN cell lines. RT-qPCR analysis was performed to verify the expression of miR-451a in IOSE80 and KGN cells. As shown in Fig. 1, the expression of miR-451a in KGN cells was significantly lower compared with that in IOSE80 cells.

ATF2 is a target gene of miR-451a, which is significantly overexpressed in KGN cells. Based on the target genes predicted by bioinformatics analysis, ATF2 was identified as a potential target of miR-451a (Fig. 2A). To confirm whether ATF2 is a target gene of miR-451a, a luciferase reporter assay was performed (Fig. 2B). Compared with the negative control, miR-451a mimic decreased WT 3'-UTR fluorescence (Fig. 2B), while MUT 3'-UTR was not altered following miR-451a transfection. Hence, the Dual-Luciferase reporter assay validated that ATF2 was a target gene of miR-451a.

To further verify the potential role of miR-451a in KGN cells, ATF2 mRNA and protein expression levels were assessed...
miR-451a negatively regulates ATF2 expression in KGN cells. (A) The miR-451a expression in KGN cells transfected with mimic control or miR-451a mimic was assessed using RT-qPCR analysis. (B) ATF2 mRNA expression in KGN cells transfected with control-plasmid or ATF2-plasmid was assessed using RT-qPCR analysis. (C and D) ATF2 mRNA and protein expression in KGN cells transfected with mimic control, miR-451a mimic, miR-451a mimic + control-plasmid or miR-451a mimic + ATF2-plasmid was detected using RT-qPCR and western blot assays. (E) The ATF2/GAPDH ratio was calculated. *P<0.01 vs. mimic control. **P<0.01 vs. control-plasmid. ***P<0.01 vs. miR-451a mimic + control-plasmid. ATF2, cyclic AMP-dependent transcription factor ATF-2; miR, microRNA. RT-qPCR, reverse transcription-quantitative PCR.

in the KGN and IOSE80 cell lines. As shown in Fig. 2C-E, ATF2 expression was significantly increased in KGN cells compared with that in IOSE80 cells.

**miR-451a inhibits KGN cell viability and induces apoptosis by reducing ATF2 expression.** To determine the functional effects of miR-451a on KGN cell growth in vitro, KGN cells were transfected with mimic control, miR-451a mimic, ATF2-plasmid, control-plasmid, miR-451a mimic + control-plasmid or miR-451a mimic + ATF2-plasmid. RT-qPCR analysis was performed to confirm transfection efficiency. As shown in Fig. 3A, miR-451a mimic significantly increased miR-451a expression levels in KGN cells compared with the mimic control group. ATF2-plasmid significantly enhanced ATF2 mRNA expression in KGN cells compared with the control-plasmid group (Fig. 3B). Simultaneously, compared with the mimic control group, miR-451a mimic significantly decreased mRNA and protein expression of ATF2 in KGN cells, and this decrease was reversed by ATF2-plasmid co-transfection (Fig. 3C-E).

To further confirm the functional association between ATF2 and miR-451a, KGN cells were transfected with mimic control, miR-451a mimic, miR-451a mimic + control-plasmid or miR-451a mimic + ATF2-plasmid. Cell viability and apoptosis was evaluated by MTT and flow cytometry assays, respectively. As shown in Fig. 4A-C, miR-451a mimic significantly decreased KGN cell viability and induced apoptosis compared with the mimic control group, and these effects were significantly reversed by ATF2-plasmid co-transfection.

Western blotting and RT-qPCR analysis were performed to detect cyclin D1 protein and mRNA expression, respectively. As shown in Fig. 5A-C, compared with mimic controls, miR-451a mimic significantly reduced the expression of cyclin D1 at both the protein (Fig. 5A and B) and mRNA (Fig. 5C) levels in KGN cells, and these effects were significantly reversed by ATF2-plasmid co-transfection. Caspase-3 activity was assessed using a kit and the protein expression of cleaved caspase-3 and pro-caspase-3 was investigated by western blotting. The results demonstrated that miR-451a mimic significantly increased the activity of caspase-3 (Fig. 5D), enhanced cleaved caspase-3 protein levels, decreased the protein expression of pro-caspase-3 (Fig. 5E) and upregulated the cleaved caspase-3/pro-caspase-3 ratio (Fig. 5F) in KGN cells. In addition, these effects were notably reversed by ATF2-plasmid co-transfection. The results indicated that miR-451a inhibited KGN cell proliferation and induced apoptosis by reducing the expression of ATF2.

**Discussion**

The results of the present study demonstrated that miR-451a is downregulated in KGN cells and that miR-451a upregulation may inhibit GC proliferation, which may be the cause underlying the development of abnormal follicles in PCOS.
expression of miR-451a was found to be significantly lower in KGN cells compared with that in IOSE80 cells. Based on previous studies, miR-451a is considered to play a role in the control of the cell proliferation, apoptosis and metastasis, by acting as a tumor suppressor (18-23). However, few studies have investigated the function of miR-451a in ovarian GCs. The present study determined that miR-451a may be a GC proliferation inhibitor.

Consistently with the results of a previous study (19), our further experiments revealed that ATF2 was a target of miR-451a. Based on Dual-Luciferase reporter gene assay results, miR-451a was shown to directly target the 3'-UTR of ATF2. Furthermore, transfection with miR-451a mimics reduced the protein and mRNA levels of ATF2 in KGN cells, whereas ATF2-plasmid transfection reversed these effects. These results indicated that miR-451a and its target ATF2 may jointly regulate the proliferation of GCs in PCOS.

ATF2 has been investigated in the context of several developmental and pathological conditions (24). Recent studies demonstrated that ATF2 may act as both a classic tumor suppressor (25) and as an oncogene (26). Moreover, it was reported that miR-451a suppressed cell migration and invasion in non-small cell lung cancer through targeting ATF2 (19). However, few studies have investigated ATF2 in ovarian GCs, and its mechanism of action remains unclear. ATF2 expression varies among different diseases. A previous study has shown that ATF2 is involved in the transcriptional regulation mechanism of mouse placental trophoblast giant cells and rat ovarian GCs in primary cultures (27). The present findings are consistent with these previous studies, whereby inhibition of ATF2 can inhibit GC proliferation.

The differences in miR-451a expression and cell proliferation observed in the present study may be attributed to differences in enzyme activity and protein and mRNA expression. The results of the present study demonstrated that miR-451a mimic significantly reduced the expression of cyclin D1 protein and mRNA in KGN cells, while it concurrently significantly increased the activity of caspase-3, increased cleaved caspase-3 and reduced the protein expression of pro-caspase-3. These effects were significantly

Figure 4. miR-451a inhibits KGN cell proliferation and induces apoptosis by reducing ATF2 expression. (A) MTT assay was performed to detect KGN cell viability. (B) Flow cytometry was performed to detect KGN cell apoptosis. (C) Cell apoptosis rates. *P<0.01 vs. mimic control. **P<0.01 vs. miR-451a mimic + control-plasmid. ATF2, cyclic AMP-dependent transcription factor ATF-2; miR, microRNA.
reversed by ATF2-plasmid co-transfection. Cyclin D1 is a key regulatory protein that promotes the transition through the restriction point in the G1 phase to S phase. Caspase-3 has been reported to be an important molecule in promoting various types of apoptosis (28). Its activation leads to the initiation of the caspase cascade, which is responsible for cell death (29). Zhen et al demonstrated that the regulatory mechanism of CCAAT/enhancer-binding protein (CEBP) in the cell cycle and steroid synthesis was inhibited in porcine ovaries. Additionally, the expression of cell cycle-related genes (cyclin D1, cyclin B1 and cyclin A1) was suppressed, indicating that knocking out the CEBP gene may inhibit apoptosis (30). The present results were consistent with those of previous studies. By targeting ATF2 in PCOS, miR-451a could decrease the expression of cyclin D1 and upregulate the activity and expression of cleaved caspase-3. However, the signaling pathway through which miR-451a/ATF2 regulate the proliferation and apoptosis of ovarian GCs remains largely unknown, and more in-depth investigation is required. Future research will focus on further exploring the molecular mechanisms underlying the regulatory effects of miR-451a/ATF2 on ovarian GCs and elucidating the related signaling pathways.

In summary, the results of the present study demonstrated that miR-451a regulated the proliferation and apoptosis of ovarian GCs by targeting ATF2, thereby providing novel insight into GC dysfunction in PCOS, and indicating miR-451a/ATF2 as a novel potential target for PCOS treatment.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

TY contributed to the conception and design of the study and the manuscript preparation. LW, YZ and JZ contributed to the data acquisition, analysis and interpretation. LL contributed...
to the data acquisition and analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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