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MiR-222 promotes the progression of polycystic ovary syndrome by targeting p27 Kip1

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ABSTRACT

Polycystic ovary syndrome (PCOS) is one of the most complex and common reproductive and endocrinologic disorders in the child-bearing age of women. Recently, miR-222 were reported to be associated with the etiology of PCOS. However, the function of miR-222 during the pathogenesis of PCOS remains unclear. In the present study, we aimed to investigate the role of miR-222 in PCOS. Firstly, miR-222 expression was examined by quantitative real-time PCR (qRT-PCR) in PCOS. The effects of miR-222 on proliferation, apoptosis and cell cycle in KGN cells were analyzed by CCK-8 assay and flow cytometry analysis, respectively. In addition, bioinformatics analysis was used to predict the target genes of miR-222, and dual-luciferase reporter assay was applied to verified the interaction between miR-222 and p27 Kip1 in KGN cells. Moreover, the expressions of p27 Kip1 in KGN cells treated with miR-222 mimics or miR-222 inhibitor were evaluated by qRT-PCR and western blot assays. The results showed that the expression of miR-222 was remarkably upregulated in PCOS tissues compared with corresponding normal tissues. In the gain-of-function and loss-of-function assays, we revealed that miR-222 mimics significantly promoted cell proliferation, while miR-222 inhibitor induced cell apoptosis and cell cycle arrested. Furthermore, p27 Kip1 was identified as a target gene of miR-222, and could be negatively regulated by miR-222 mimics in KGN cells. In conclusion, our findings suggested that miR-222 may promote the progression of PCOS by targeting p27 Kip1.

1. Background

Polycystic ovary syndrome (PCOS) characterized by hyperandrogenism, polycystic ovaries and anovulation, is one of the most complex and common endocrinologic disorders in the child-bearing age of women; PCOS accounts for around 6–10% of the female population in the world [1–3]. In addition, more than 20% of female with infertility problems have been demonstrated to be associated with PCOS [3,4]. Moreover, PCOS was reported to increase the risk of multiple of human diseases including insulin resistance (found in more than 60% of PCOS patients), type 2 diabetes mellitus (T2DM), cardiovascular disorders and endometrial tumors [5,6]. And PCOS was even revealed to exert psychological burdens on life, leading to anxiety and depression [7]. Although the cause of PCOS remains largely unclear, increasing evidences suggested that it may be the consequence of the interaction between environmental and genetic factors [8,9]. Many researchers attempted to elucidate the mechanisms underlying the pathogenesis of PCOS. However, little was known about the etiology of PCOS so far.

MicroRNAs (miRNAs) are small noncoding single-stranded RNA molecules with 18–24 nucleotides, which acted as a gene expression regulator during the posttranscriptional process [10]. It was documented that miRNAs exhibited their gene regulatory effects by directly binding to the 3′- untranslated region (3′-UTR) of targeted mRNA [11]. Through regulation of gene expression, miRNAs are involved in multiple biological course including cell proliferation, survival and apoptosis. In addition, miRNAs are associated with many human diseases including metabolic disorders, neurodegenerative disorders and human cancers [11]. Recently, miRNAs were reported to participate in the pathogenesis of PCOS [12,13]. For instance, Wan Q et al. indicated that the reduced miR-16 expression in PCOS could inhibit the proliferation of ovarian granulosa cells by targeting programmed cell death proteins [4,14]. Additionally, miR-222 were reported to be closely associated with the etiology of PCOS. However, the function of miR-222 in the pathogenesis of PCOS remains unclear [12,15].

In the present study, we aimed to explore the role of miR-222 during the pathogenesis of PCOS. Firstly, we assessed the expression level of miR-222 in PCOS tissues and corresponding normal tissues. Then, the effect of miR-222 on the proliferation, apoptosis and cell cycle in KGN

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cells were evaluated.

The novelty of current study is that bioinformatics analysis and dual-luciferase reporter assay were performed to predict and verify the target genes of miR-222 in KGN cells.

2. Materials and methods

2.1. Clinical specimens and cell culture

A total of twenty paired PCOS tissues and corresponding normal tissues were collected in the second affiliated hospital of Fujian Medical University from Jan, 2017 to Mar, 2018 according to the principles of the Declaration of Helsinki. Written informed consents were provided by all patients and all manipulates in the present study were approved by the ethics committee of The Second Affiliated Hospital of Fujian Medical University (No.2017109). The human granulosa-like tumor cell line KGN was used in this study, because it possesses the majority of physiological characteristics of ovarian cells. KGN cells were obtained from RIkagaku KENkyusho/Institute of Physical and Chemical Research (RIKEN, Japan, RCB1154), and cultured in the Dulbecco's Modified Eagle Medium (DMEM) medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Gibco) with 100 U/mL penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were culture in a humidified atmosphere (5% CO_2 and 95% air) at 37℃.

2.2. Cell proliferation analysis

The effects of miR-222 on cell proliferation was determined by Cell Counting Kit-8 (CCK-8) assay kit (Dojindo, Kumamoto, Japan) according to the instructions of manufacturers. In brief, KGN cells were seeded into 96-well plates at a concentration of 2×10^4 cells/well and cultured at 37℃ overnight. After eight hour miR-222 mimics or inhibitor transfection, KGN cells were cultured at 37℃ for three days. Meanwhile, at 0, 24, 48 and 72 h time point, the cells were incubated with CCK-8 reagent for another 2 h, and the absorbance was determined at 450 nm by a microplate reader. MiR-222 mimics (5′-AGCUACAUC UGGCUACUGGGU-3′) and inhibitor (5′-UCGAUGUAGACCGAUGAC CCA-3′).

2.3. RNA extraction and quantitative real-time PCR (qRT-PCR assay)

Total RNAs of treated KGN cells were prepared using Trizol reagent (Invitrogen) according to the protocols provided by the manufacturers; 50 ng of total RNAs were used to synthesize cDNA with the Reverse Transcription kit (Takara, Tokyo, Japan). The amplification of miR-222 and p27 Kip1 were performed using the BestarTM qPCR MasterMix (DBI Bioscience, Shanghai, China) following the protocols obtained from the manufacturers on the ABI PRISM 7500 system (Thermo Fisher Scientific, Waltham, MA, USA). The sequence of primers used in this study were showed as follow: GAPDH: F, 5′-TGTTCGTCATGGGTGTG AAC-3′, R, 5′-ATGGCA TGGACTGTGGTCAT -3′; U1: F, 5′- GGGAGATA CCATGATCACGAAGGT', R, 5′ − CCACAAATTATGCAGTCGAGTTTC CC-3′; miR-222: F, 5′- CGCAGCTAC ATCTGGCTACTG-3′, R, 5′-GTGCAGG GTCCGAGG T-3′; p27 Kip1: F, 5′-ATG TCAAACGTGCGAGTGTCTAA-3′, R, 5′-TTACGTTTGACGTCTTCTGAGG-3′. The expression of p27 Kip1 was normalized to GAPDH, and miR-222 expression was normalized to U1.

2.4. Oligonucleotide transfection

The miR-222 mimics and inhibitor, as well as scramble negative control were all obtained from GenePharma (Shanghai, China). KGN cells were seeded into 6-well plates at a concentration of 2×10^6 cells/well. After cultured overnight at 37℃, KGN cells were incubated with miR-222 mimics, inhibitor, or scramble negative control using the Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) following the instructions provided by the manufacturers.

2.5. Ki-67 staining

Nuclear protein Ki-67 is associated with cellular proliferation, which is regarded as a cellular marker for proliferation. The miR-222 mimics or inhibitor treated KGN cells were cultured in Lab-Tek chamber slides (Nunc, Naperville, IL), and fixed with 4% paraformaldehyde for 15 min at room temperature. After washed with pre-cold phosphate buffer (PBS) for three times, treated KGN cells were subjected to incubate with 1% bovine serum albumin (BSA) containing primary antibody Ki-67 (1:3000, Novocastra, Newcastle, UK) at 4℃ for 8 h. Subsequently, KGN cells were incubated with an FITC-labeled secondary antibody (1:200, Abcam Cambridge, MA, USA) in 1% BSA for 2 h. After washing with PBS, cells were stained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 10 min. Finally, Ki-67 positive cells were counted under a microscope.

2.6. Cell cycle and apoptosis analysis

Flow cytometry analysis was used to analyze the cell cycle and apoptosis of KGN cells. Briefly, the KGN cells were harvested and washed with PBS for twice. Then, collected cells were fixed in 70% ethanol for 3 h. After washed with PBS for twice, cells were incubated with Annexin V-isothiocyanate (FITC) and PI (Keygentec, Shanghai, China) for 10 min at room temperature. Early apoptotic cells (PI negative, FITC Annexin V positive); Late apoptotic cells (PI positive, FITC Annexin V positive). Finally, cell cycle and apoptosis were analyzed using a FACScan instrument (Becton Dickinson, Franklin Lakes, NJ, USA).

2.7. Dual-luciferase reporter assay

The fragments of p27 Kip1 containing the wide type or mutant putative miR-222 binding site were sub-cloned into the luciferase vector psi-CHECK2 (Promega, Madison, WI, USA) to establish recombinant reporter plasmid, named as p27 Kip1-WT or p27 Kip1-MT, respectively. KGN cells (1×10^5 cells/well) were seeded into 24-well plates and maintained at 3℃ overnight. Subsequently, KGN cells were co-transfected p27 Kip1-WT or p27 Kip1-MT with miR-222 mimics or its negative control using lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) under the instruction provided by the manufacturers. Two days after co-transfection, KGN cells were collected and luciferase activity of each group was measured by Dual Luciferase Assay System (Promega, Madison, WI, USA).

2.8. Western blot assay

Total proteins of KGN cells were extracted by RIPA buffer (Sigma Aldrich, R0278, St. Louis, MO, USA). The cell supernatants were collected after centrifugation (12,000 rpm/min) at 4℃ for 20 min, and the concentration of total proteins was determined by a BCA kit (Pierce, Rockford, IL, USA). After isolated with 10% SDS-PAGE, targeted proteins were transferred into nitrocellulose membranes (Millipore, Billerica, MA, USA). The non-specific plots were blocked by incubating membranes with 5% low fat dried milk for 2 h. Primary antibodies that against p27 Kip1 (Rabbit, 1:2000, ab92741, Abcam), and β-actin (Rabbit, 1:2000, ab5694, Abcam) were used to incubate with membranes overnight. And horseradish peroxidase conjugated donkey-anti-rabbit secondary antibodies were applied to incubate with membranes for another 2 h. The signals were detected by enhanced chemiluminescent reagents.

2.9. Statistical analysis

Data from the present study were expressed as Mean ± SD, the difference between two groups was analyzed by student *t*-test, and one-

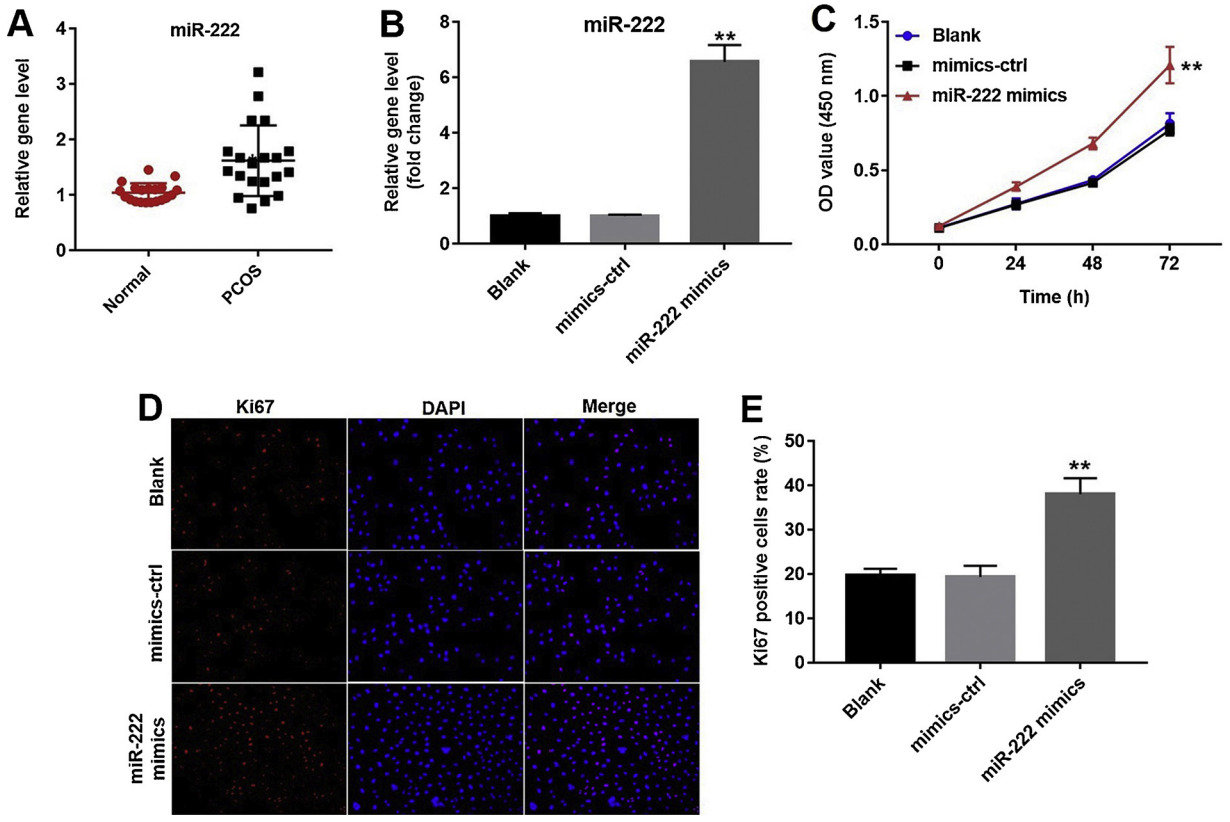

Fig. 1. MiR-222 level was upregulated in PCOS and overexpression of miR-222 promoted cell proliferation. (A) The relative expression of miR-222 was detected in 20 paired normal and PCOS tissues by qRT-PCR. (B) The relative expression of miR-222 in KGN cells transfected with miR-222 mimics and its negative control were assessed by qRT-PCR. (C) CCK-8 assay was applied to evaluate the effect of miR-222 mimics on cell proliferation. (D and E) Ki-67 staining was carried out to further assess the effect of miR-222 mimics or its negative control on the proliferation of KGN cells. $^{**}P < 0.01$.

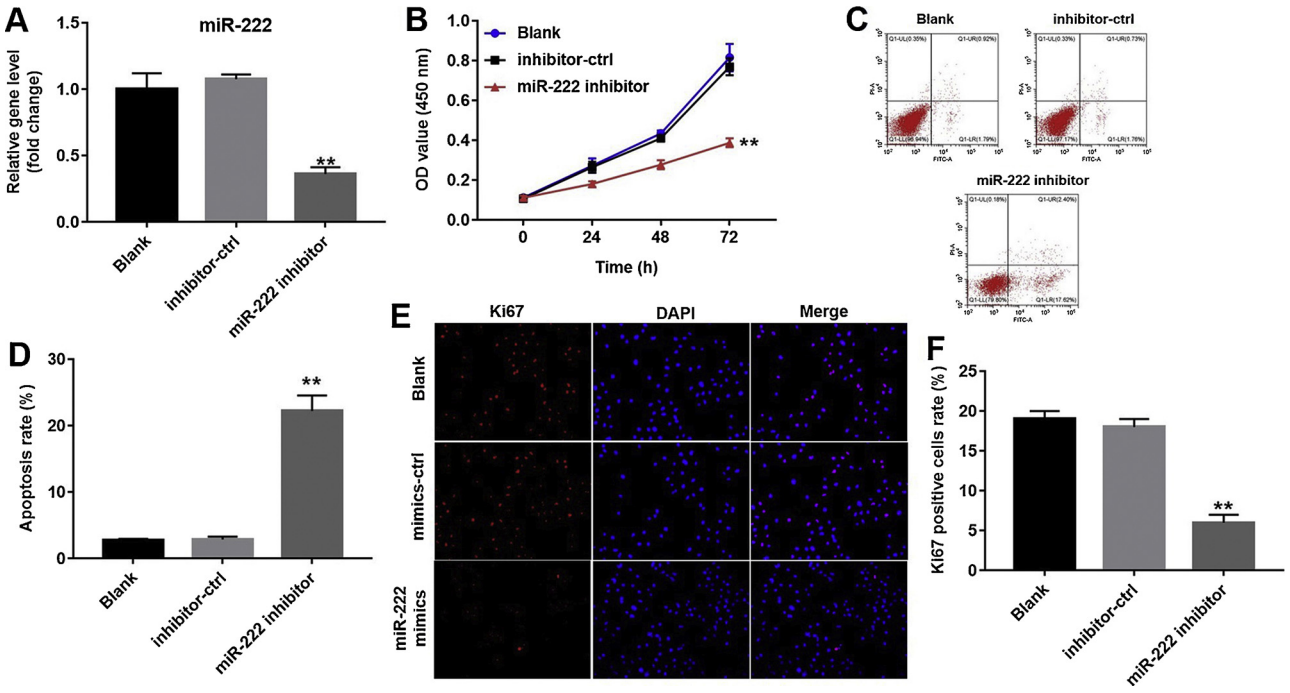

Fig. 2. Downregulation of miR-222 inhibited KGN cell proliferation via promoting apoptosis. (A) The relative expression of miR-222 in KGN cells transfected with miR-222 inhibitor and its negative control were assessed by qRT-PCR. (B) The cell proliferation of KGN cells transfected with miR-222 inhibitor and its negative control were examined by CCK-8 assay. (C and D) Flow cytometry analysis was performed to detect the apoptosis rate in miR-222 inhibitor and its negative control treated KGN cells. (E and F) Ki-67 staining was applied to determine the proliferation in miR-222 inhibitor treated KGN cells. $^{**}P < 0.01$.

way ANOVA followed by Dunnett's test was used for statistical analyses in more than two groups. P value less than 0.05 was considered statistically significant.

3. Results

3.1. The level of MiR-222 was upregulated in PCOS and overexpression of miR-222 promoted proliferation of KGN cells

To explore the potential role of miR-222 in PCOS, we firstly examined the level of miR-222 in 20 paired PCOS tissues and corresponding normal tissues by qRT-PCR. The results showed that miR-222 expression was significantly increased in PCOS tissues compared with normal tissues (**$P < 0.01$, Fig. 1A). To further investigate the biological functions of miR-222 in PCOS, KGN cells were transfected with miR-222 mimics. The efficiency of miR-222 mimics transfection was examined by qRT-PCR and the results indicated a significant upregulation of miR-222 in miR-222 mimics treated KGN cells compared with those cells treated with noting (blank) (**$P < 0.01$, Fig. 1B). In additio, CCK-8 assay indicated miR-222 mimics significantly increased cell proliferation compared with mimics-control group in CCK-8 assay (**$P < 0.01$, Fig. 1C). Moreover, the Ki-67 positive rate in miR-222 mimics treated KGN cells was significantly higher than those cells treated with mimics-control (**$P < 0.01$, Fig. 1D and E). All these data suggested overexpression of miR-222 promoted the proliferation of KGN cells.

3.2. Downregulation of miR-222 inhibited proliferation of KGN cells via promoting apoptosis

We next transfected KGN cells with miR-222 inhibitor and examined the efficiency of transfection using qRT-PCR. As indicated in Fig. 2A that the level of miR-222 was notably downregulated in miR-222 inhibitor transfected cells compared to those cells transfected with the negative control (inhibitor-control) (**$P < 0.01$). In addition, miR-222 inhibitor significantly decreased the cell proliferation via inducing apoptosis (**$P < 0.01$, Fig. 2B–D). Moreover, miR-222 inhibitor markedly decreased Ki-67 positive rate in KGN cells compared with inhibitor-control groups (**$P < 0.01$, Fig. 2E and F).

3.3. p27 Kip1 was the target of miR-222 in PCOS

It was well documented that miRNAs may exhibit their biological effects through regulation of targeted genes by binding to the 3′-untranslated regions (3′-UTR) of mRNA. Therefore, we predicted the targeted gene of miR-222 by using bioinformatics analysis and the results indicated putative binding site of miR-222 in the 3′-UTR of p27 Kip1 (Fig. 3A). In order to verified the interaction between miR-222 and p27 Kip1, we sub-cloned the p27 Kip1 fragment containing the wide type or mutant miR-222 binding site into the luciferase reporter plasmid psi-CHECK2 to form the p27 Kip1-wide type (p27 Kip1 WT) or p27 Kip1-mutant (p27 Kip1 MT) recombinant plasmid (Fig. 3B). In the luciferase reporter assay, we found that the luciferase activity driven by p27 Kip1 WT was notably attenuated by miR-222 mimics (**$P < 0.01$, Fig. 3C). In addition, the expression of p27 Kip1 was significantly decreased in PCOS tissues compared with normal tissues (**$P < 0.01$, Fig. 3D). All these data suggested p27 Kip1 was the target of miR-222 in PCOS.

3.4. Overexpression of miR-222 significantly downregulated the expression of p27 Kip1 in KGN cells

We next evaluated the expression of p27 Kip1 in miR-222 mimics-treated KGN cells by qRT-PCR and western blot assays. The results showed that the relative mRNA expression of p27 Kip1 was markedly decreased in miR-222 mimics transfected KGN cells compared with those cells transfected with mimics-control (Fig. 4A). In addition, western blot assay also confirmed the expression of p27 Kip1 was remarkably inhibited by miR-222 mimics compared with mimics-control treated groups (Fig. 4B and C).

3.5. Inhibition of miR-222 remarkably upregulated the expression of p27 Kip1 and induced G1/S phase cell cycle arrest in KGN cells

Since p27 Kip1 plays an important role during cell cycling, the effect of miR-222 on cell cycling was then evaluated. We found miR-222 inhibitor significantly enhanced the expression of p27 Kip1 in both gene and proteins levels (Fig. 5A–C). In addition, apoptosis-related proteins Bax and active caspase 3 were increased by miR-222 inhibitor in cells (Fig. 5B and C). This result was consistent with apoptosis assay. Moreover, the cell cycle analysis revealed that the cell number in G0/G1 phase was significantly increased, whereas cell number in S phase was remarkably decreased in miR-222 inhibitor treated group compared to the control group (Fig. 5D and E). All there outcomes illustrated inhibition of miR-222 induced G1/S phase cell cycle arrest via regulation of p27 Kip1 expression in KGN cells.

4. Discussion

The high and gradually increasing incidence of PCOS raised a widely concern in the clinical research community [16]. In the past several years, as many as several thousands of research articles were published in the world concerning the different aspects of PCOS [17]. Despite the increased focus on PCOS, the etiology of this disease has not been fully understood [3]. In addition, the diagnosis of PCOS remains highly controversial due to the entire complexity in the pathogenesis of PCOS [3]. Anovulation, hyperandrogenism, and polycystic ovaries were considered to be the key diagnostic properties of PCOS, following the instructions of Rotterdam Criteria, and patients with at least two of the three features could be diagnosed as PCOS [18]. However, there still lacks of several objective tests that could absolutely ensure the diagnosis [18]. Thus, elucidating the mechanisms underlying the etiology of PCOS could contribute to the diagnosis, treatment and prevention of PCOS.

Due to miRNAs were reported to participate in the pathogenesis of PCOS [19]. Previously studies have demonstrated that miR-222 involved in many different aspects of tumors, such as drug-resistance, prognosis, and progression [20–22]. Moreover, Jiayin Liu et al. identified miR-222 as one of the three upregulated miRNAs (miR-222, miR-146a, and miR-30c) in the serum of PCOS patients by using the TaqMan Low Density Array followed by individual qRT-PCR assay [12]. Furthermore, miR-222 in the Jiayin Liu study was predicted to be associated with the metastasis, cell cycle and endocrine [12]. In the present study, we aimed to further investigate the roles of miR-222 in PCOS. The expression of miR-222 in PCOS tissues was revealed significantly higher than normal tissues. And in the following gain-of-function and loss-of-function assays, we demonstrated miR-222 could significantly promote the cell proliferation, and suppress cell apoptosis of KGN cells, suggesting miR-222 may be a novel therapeutic target and diagnostic biomarker of PCOS.

It was well established that miRNAs may be associated with the initiation and progression of multiple human cancers through the regulation of their target genes by directly binding to them [23]. In the present study, p27 Kip1 was identified as a target gene of miR-222 in PCOS, and p27 Kip1 could be negatively regulated by miR-222, suggesting that p27 Kip1 may be a downstream gene of miR-222. The p27 Kip1 protein, encoded by the gene located in chromosome 12p13, is an important inhibitor of cyclin-dependent kinases (CDKs) [24,25]. Previously studies indicated that p27 Kip1 not only involved in the regulation of cell cycle in G1 phase, but also participated in modulation beyond the G1 phase by inhibiting CDKs [24]. It has been well documented that the aberrant expression of check point genes always be associated with tumorigenesis, such as Tp53, C-Myc, and p27 Kip1

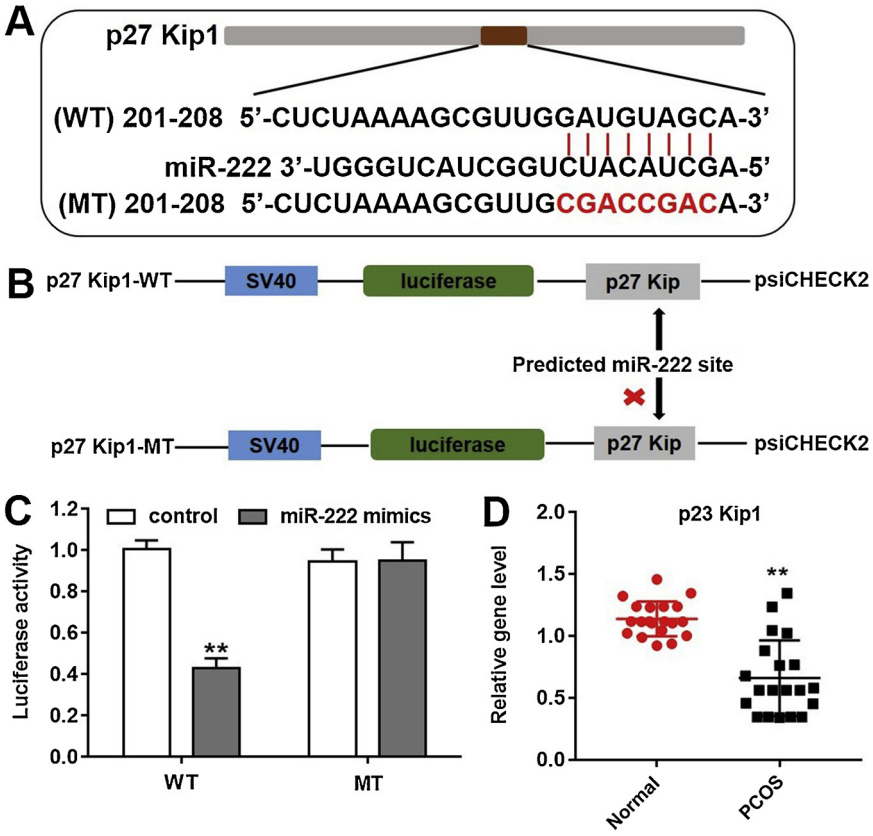

Fig. 3. p27 Kip1 was the target of miR-222 in PCOS. (A) The putative binding site of miR-222 in p27 Kip1 was predicted by bioinformatics analysis. (B) Schematic diagram of p27 Kip1 wide-type and mutant luciferase reporter plasmid construction. (C) Dual-luciferase reporter assay was used to verified the interaction between miR-222 and p27 Kip1 in KGN cells. (D) The relative expression of p21 Kip1 was detected in 20 paired normal and PCOS tissues by qRT-PCR. $^{**}P < 0.01$.

[26–28]. In addition, p27 Kip1 was reported to act as a tumor suppressor by interacting with miRNAs in several of human cancers [29]. For instance, miR-200c could promote tumor progression of gastric cancer by repressing the expression of p27 Kip1 [30], which was similar to our findings in PCOS.

In summary, increased expression of miR-222 in PCOS was demonstrated to promote KGN cell proliferation, and inhibit cell apoptosis by directly repressing p27 Kip1. These data suggest that miR-222 and p27 Kip1 may be two important novel therapeutic targets and biomarkers for PCOS patients. However, it is still need further in vivo evidences to prove their value in the diagnosis and treatment of PCOS.

Availability of data and materials

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

Authors' contributions

Xiaolan Huang, Liping She, Xiangmin Luo, Shuzhen Huang, Jinxiang Wu

Xiaolan Huang, Liping She, Xiangmin Luo and Shuzhen Huang designed and performed experiments. Jinxiang Wu performed staining assay, analyzed and interpreted the data and drafted the manuscript.

Ethics approval and consent to participate

All manipulates in the present study were approved by the ethics committee of Fujian Medical University.

Patient consent for publication

Written consents were provided by patients

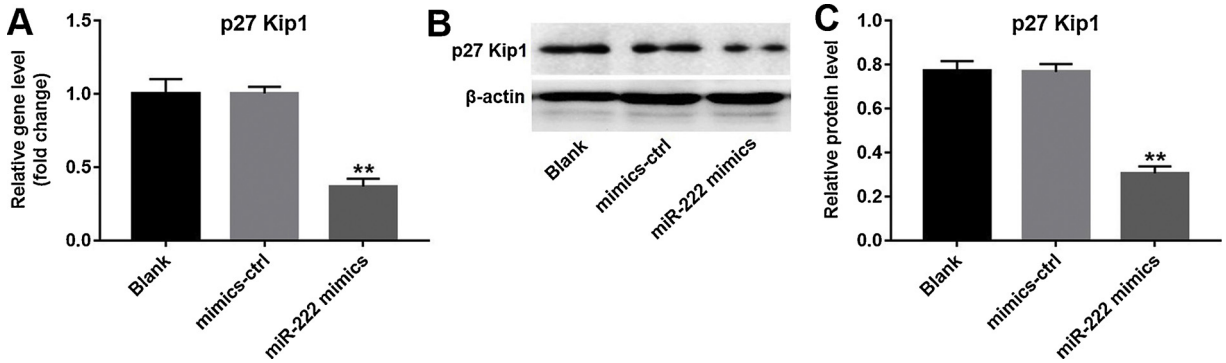

Fig. 4. Overexpression of miR-222 significantly inhibited the expression of p27 Kip1 in KGN cells. (A) The relative mRNA expression of p27 Kip1 was examined by qRT-PCR in miR-222 mimics treated KGN cells. (B and C) The relative protein expression of p27 Kip1 in miR-222 mimics treated KGN cells was assessed by western blot assay. $^{**}P < 0.01$.

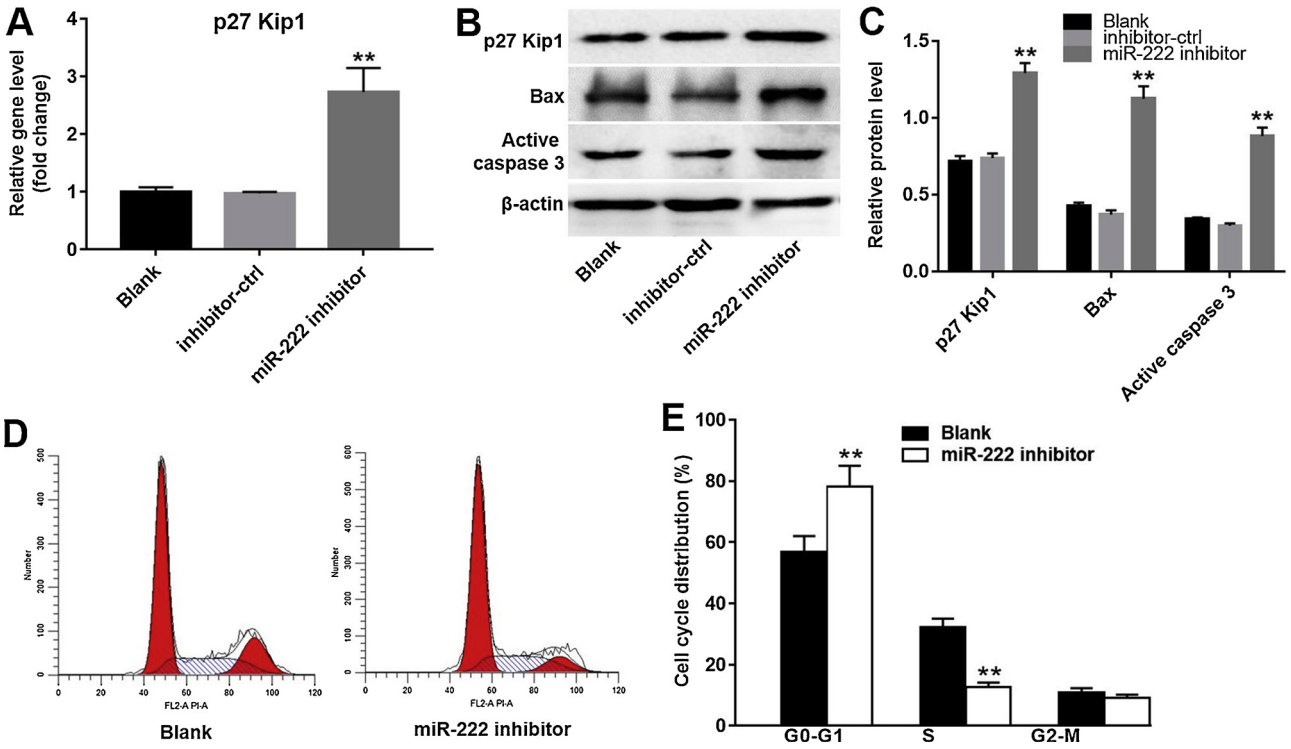

Fig. 5. Inhibition of miR-222 remarkably upregulated the expressions of p27 Kip1, Bax and active caspase 3, and induced G1/S phase cell cycle arrest in KGN cells. (A) The relative mRNA expression of p27 Kip1 was examined by qRT-PCR in miR-222 inhibitor treated KGN cells. (B and C) The relative protein expressions of p27 Kip1, Bax and active caspase 3 in miR-222 inhibitor-treated KGN cells was assessed by western blot assay. (D and E) The effect of miR-222 inhibitor on cell cycle was analyzed by flow cytometry analysis. $^{**}P < 0.01$.

Competing interests

The authors declare that they have no competing interests

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