miR-423-3p inhibits CTNNBIP1/WNT preventing hyperandrogenic PCOS

Running head: miR-423-3p and PCOS

Shanshan Zhang[1,2,*], Yajing Liu[3,*], Mingming Wang[4,5,*], Donata Ponikwicka-Tyszko[6,7], Slawomir Wolczynski[6,8], Li Chen[9], Xuan Huang[9], Bing Yao[9,#], Nafis A Rahman[7,8,#], Xiangdong Li[2,8,#]

[1] School of Life Sciences, Jining Medical University, No. 669 Xueyuan Road, Donggang District, Rizhao, Shandong Province 276826, PR China

[2]State Key Laboratory of Animal Biotech Breeding, College of Biological Sciences, China Agricultural University, Beijing, China

[3]School of Biomedical Engineering, Tsinghua University, Beijing 100084, China

[4]Department of Physiology, School of Basic Medical Sciences, Xuzhou Medical University, Xuzhou, Jiangsu Province 221009, PR

[5]National Experimental Teaching Demonstration Center for Basic Medicine, Xuzhou Medical University, Xuzhou, Jiangsu Province 221009, PR

[6]Department of Biology and Pathology of Human Reproduction, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland

[7]Institute of Biomedicine, Faculty of Medicine, University of Turku, Turku, Finland

[8]Department of Reproduction and Gynecological Endocrinology, Medical University of Bialystok, Bialystok 15276, Poland

[9]Department of Reproductive Medicine, Affiliated Jinling Hospital, Medicine School of Nanjing University, Nanjing 210002, Jiangsu, China

*Co-first author: These authors contributed equally to this work.

#Correspondence:

Xiangdong Li, State Key Laboratory of Animal Biotech Breeding, College of Biological Sciences, China Agricultural University, Beijing, China; Beijing 100193, China; Tel/Fax; 86-10-62734389; E-mail: xiangdongli68@126.com

Nafis A Rahman, Institute of Biomedicine, Faculty of Medicine, University of Turku, Turku, Finland; Department of Reproduction and Gynecological Endocrinology, Medical University of Bialystok, Bialystok 15276, Poland; E-mail: nafrah@utu.fi

Bing Yao, Department of Reproductive Medicine, Affiliated Jinling Hospital, Medicine School of Nanjing University, Nanjing 210002, Jiangsu, China; E-mail: yaobing@nju.edu.cn

DISCLOSURE SUMMARY: The authors have nothing to disclose.

Summary sentence:

miR-423-3p may play a novel role in hyperandrogen polycystic ovary syndrome (PCOS) progression, serving as a potential serum biomarker and therapeutic target. It helps prevent hyperandrogenic PCOS by inhibiting the CTNNBIP1/WNT signaling pathway.

Funding information

This work was supported by grants from Shandong Province Medical Health Science and Technology Project (Grant No. 202402010756); the Shandong Provincial University Youth Innovation Team, China (No. 2024KJG031); the National Natural Science Foundation Program (Grant No. 31970802); Beijing Natural Science Foundation Program (Grant No. 7202099); National Natural Science Foundation of China (Grant No. 82401923); Natural Science Research of the Jiangsu Higher

Education Institutions of China (Grant No. 22KJB180007); the Outstanding Talent Research Funding of Xuzhou Medical University (Grant No. RC20552029); B.SUB.23.305 from the Medical University of Bialystok, Poland and the 2115 Talent Development Program of ChinaAgricultural University.

Abstract

Polycystic ovary syndrome (PCOS) lacks the generally accepted diagnostic biomarkers and targeted therapy. Increasing evidence indicates that microRNAs (miRNAs) play a crucial role in PCOS. Hereby, we tested the functional implications of a novel miRNA (miR-423-3p) as a mediator in the progress of hyperandrogenic PCOS, as well as its potential as a new serum biomarker and therapeutic target for the PCOS. We found significantly decreased miR-423-3p levels in serum, granulosa cells (hGCs), and follicular fluid (FF) of PCOS patients (n=40) compared to healthy controls (n=30), and this decrease corroborated in PCOS-like mouse models. The receiver operating characteristic (ROC) curve analysis for circulating miR-423-3p indicated high diagnostic potential as a biomarker, with an area under the curve (AUC) of 82%. miR-423-3p influenced human granulosa cell (KGN) proliferation by directly targeting CTNNBIP1 modulated WNT signaling pathway. We further proved as mechanistic role that the elevated dihydrotestosterone (DHT) inhibited the expression of miR-423-3p via the activation of the androgen receptor, and the overexpression of miR-423-3p normalized the function of androgen-induced GCs. While we overexpressed miR-423-3p, it counteracted androgen-induced dysfunction in GCs. Antiandrogen treatment restored the reproductive phenotypes in letrozole-induced PCOS-like mice and regulated miR-423-3p expression and its downstream effects. Ovarian intrabursal injection of miR-423-3p antagomir in wildtype (WT) mice induced PCOS-like phenotypes, further underscoring its functional role. Our results demonstrated that miR-423-3p emerged as a novel mediator in hyperandrogenic PCOS progression and it holds

promise as both a diagnostic biomarker and a therapeutic target.

Keywords: miR-423-3p, polycystic ovary syndrome, proliferation, biomarker

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine diseases affecting up to 6–20% of reproductive-aged women, mainly characterized by two of the following three criteria: polycystic ovarian morphology, hyperandrogenism, and chronic anovulation [1, 2]. The ovarian follicular microenvironment and maternal signals, mediated mostly by the granulosa cells (GCs), are responsible for follicle growth initiation, oocyte maturation, and atresia [3, 4]. Dysfunction of GCs in patients with PCOS, including decreased proliferation, increased apoptosis, or unbalanced hormone production, may contribute to abnormal folliculogenesis [5, 6].

MicroRNAs (miRNAs) are 21-25-nucleotide non-coding RNAs that can post-transcriptionally regulate the gene expression by binding specifically to the 3'untranslated region (UTR) of the target genes [7]. miRNAs have been found to play a crucial role in various diseases[8, 9]. In previous studies, we have found that miRNAs were involved in the pathogenesis of PCOS by mediating hormone secretion [10], ovarian granulosa cell proliferation, and apoptosis [11]. Therefore, revealing the biological function of miRNAs in the process of PCOS is beneficial for the diagnosis, treatment and understanding of the pathogenesis of PCOS.

In this study, by filtering the published databases and combined with a series of experimental verifications, we found that miR-423-3p was significantly decreased in the serum, human granulosa cells (hGCs), and follicular fluid (FF) samples of both PCOS patients and PCOS-like mouse models. We hypothesized that miR-423-3p might be a novel mediator during the progression of PCOS. To test our hypothesis, we

analyzed the function of miR-423-3p by using human GC cell line and mice models to explore the mechanistic role of miR-423-3p in the dysfunction of GCs. Our findings may provide a better understanding of miR-423-3p and its potential novel diagnostic effects in PCOS.

Methods

Participants

Before conducting the experiment, the required clinical sample size for this study were calculated by using PASS 11 (NCSS, USA). A total of 70 cases were recruited, including 40 patients with PCOS diagnosed on the basis of the Rotterdam criteria [12] and 30 women with no history of reproductive disorders as a normal group. The control group consisted of women with regular menstruation and no hyperandrogenization who underwent an *in-vitro* fertilization (IVF) program due to the male factor. hGCs and follicular fluid (FF) were collected from patients who underwent IVF treatment at the Department of Reproduction and Gynecological Endocrinology, at the Medical University of Bialystok, Poland. Seum samples were collected from all participants after a 12 h overnight fasting. Concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol (E_2), prolactin (PRL), anti- Müllerian hormone (AMH), testosterone (T), insulin, sex hormone-binding globulin (SHBG), dehydroepiandrosterone sulfate (DHEA-S) were assessed by immunoradiometric assays. Anthropometric variables such as age, body mass index (BMI), and the levels of FSH, LH, T, E_2, AMH, etc., and the biochemical parameters were recorded in Table 1.

The research was based on the ethical principles of the Declaration of Helsinki. All clinical samples were collected according to protocols approved by the local Human Investigation Ethics Committee (license numbers APK.002.38.2021). The informed consent was signed by each patient for the collection and use of their samples in this

study.

Clinical sample preparation

hGCs and FF were collected at the time of the follicle aspiration. Briefly, after oocyte removal, FF containing hGCs were pooled from the follicles and centrifuged at 250 × g for 10 min. FF were centrifuged to remove cells and stored at −80℃. The fraction containing hGCs was carefully aspirated, transferred to a new centrifuge tube, resuspended in phosphate-buffered saline (PBS) and centrifuged at 250 × g for 5 min. hGCs were collected and frozen at −80°C until analyzed.

RNA extraction and real-time quantitative reverse transcription-PCR (qRT-PCR)

The total RNA was extracted by TRIzol (Invitrogen, USA), and cDNA was obtained with a reverse transcription kit. The targeted gene expression was measured by qRT-PCR using the SYBR Premix Ex Taq (Takara, Japan). *Glyceraldehyde phosphate dehydrogenase* (*GAPDH*) was taken as the internal reference (U6 was taken as the internal reference). The $2^{-\Delta\Delta Ct}$ method was applied to analyze mRNA expression levels. The primer sequences of the tested genes are listed in Table S2.

Biomarker validation

A total of 48 patients were recruited, including 28 patients with PCOS and 20 non-PCOS women. The diagnosis of PCOS is based on the Rotterdam consensus. The exclusion criteria included pregnant women, malignant tumor history, other diseases that could cause similar symptoms of PCOS (hyperthyroidism, Cushing syndrome and hyperprolactinemia, etc.), or treatment with steroid hormone drugs or herbal medications with known or uncertain interactions with steroid hormone within three

months. The Non-PCOS women were those who had menstrual abnormalities and the further examination results of biochemical tests and B-ultrasound were not consistent with the diagnosis of PCOS. The Non-PCOS women also met the following exclusion criteria: previous ovary surgery, malignant tumor history, severe dysfunction of important organs or treatment with the drugs that may influence the steroid hormone. Written informed consent was obtained from all participants prior to testing. The blood sample from each participant was collected on the 2–5th day of menstruation. Key clinical characteristics of this validation cohort, including hormonal profiles and anthropometric data, are summarized in Supplementary Table 1. The experiments adhered to ethical guidelines approved by the Medical Ethics Committee of Affiliated Jinling Hospital, Medical School of Nanjing University (Approval No. 2024DZGJJ-013). Serum and hGCs were collected using protocols identical to those described as above. cDNA synthesis was performed using the TaqMan®MicroRNA Reverse Transcription Kit (Applied Biosystems, USA). TaqMan miRNA probes (cel-miR-39, # 000200; RNU6B, #001093; miR-335-5p, #000543; Applied Biosystems, USA) were used to assess the selected miRNA expression profile. Quantitative real-time PCR (qPCR) assays were performed in a total 20 μL reaction volume with TaqMan Universal PCR Master Mix, no AmpErase UNG (Applied Biosystems, USA). The miRNA Ct-values were normalized using the exogenous cel-miR-39 and RNU6B.

Cell culture

The hGC line KGN and mGCs were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F12) (Gibco, USA) containing 10% charcoal-

stripped fetal bovine serum (Thermo Fisher Scientific, USA) and 1% antibiotics (mixture of penicillin, streptomycin, and neomycin; Gibco) in a 37°C, 5% CO2 incubator (Thermo Fisher Scientific, USA). Cells were passaged every 3 days. KGN cells were purchased from Wuhan Pricella.

Establishment of the PCOS-like mouse models

C57BL/6J mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. China, and housed in quarters controlled for temperature (25 °C) and light (12 h, 12 h dark). Mice were provided with non-purified commercial chow diet (Beijing Hfk Bioscience Co., Ltd. China) and fresh water *ad libitum*. All the procedures were approved by the Ethics Committee for Animal Experimentation of China Agricultural University (license numbers: AW62201202-3-7).

To establish the DHEA-induced PCOS mouse model, 3-week-old female mice were subcutaneously injected daily with DHEA (6 mg/100 g body weight) dissolved in sesame oil for 28 consecutive days, with only sesame oil injection as the control group [13].

To establish the letrozole-induced PCOS mouse model, 3-week-old female mice were treated daily with letrozole (1 mg/kg body weight) dissolved in 1% carboxymethylcellulose (CMC) daily by oral gavage for 28 consecutive days, the control group received the vehicle only (1% aqueous solution of CMC) once daily [14].

The DHT-induced mouse model was established using a previously published methodology [15]. Briefly, adult C57BL/6J females were paired with males at 16: 00 in the afternoon. Mice with vaginal plugs were identified the next morning and housed

individually. Pregnant mice were injected subcutaneously with sesame oil (control group) or sesame oil containing 200 μg of DHT on days 16, 17, and 18 of pregnancy. The three-month-old female adult offspring of the mice and control mice were then observed throughout their postnatal lives.

After the model was established, all mice were anesthetized with pentobarbital sodium (50 mg/kg) injected intraperitoneally, and blood was obtained by cardiac puncture.

Antiandrogen treatment in the letrozole-induced mouse model of PCOS

Three-week-old female mice were intragastrical injected daily with letrozole (1 mg/kg body weight) dissolved in 1% CMC (2 mL/kg) and flutamide (5 mg/kg body weight) for 28 consecutive days. All mice were anesthetized with pentobarbital sodium (50 mg/kg) injected intraperitoneally, and blood was obtained by cardiac puncture.

miRNA injection technique

After isoflurane anesthesia, the back skin and muscles of mouse were incised and both ovaries were carefully pulled out and the intracapsular micor-injection was performed under stero-microscopic magnification. Mice were micro-injected with a micro-syringe (7653-01, Hamilton, USA). After injection, the treated mice were housed separately, and the estrous cycle was assessed by the vaginal cytology at 11 days after the injection. Mice sera were collected for the E_2 and T analyses. The miR-423-3p antagomir and miR-NC were purchased from Shanghai GeneBio (China).

Estrous cycle assessment and ovarian histology

Estrous cycle was assessed by the vaginal cytology. Female mice were sacrificed

and their ovaries were dissected for RNA/protein extraction. The specimens were fixed in 4% paraformaldehyde for 72 h. Ovaries were sectioned at a thickness of 5 μm and stained with hematoxylin and eosin.

Plasmid construct

To determine whether miR-423-3p targets CTNNBIP1, the entire 3'UTR of the human CTNNBIP1 gene was cloned into the psiCHECK-2 dual luciferase reporter vector (Promega, USA) by the endonuclease sites XhoI and NotI to established a CTNNBIP1 3'UTR plasmid. The deletions of the predicted seed regions of miR-423-3p within the human CTNNBIP1 3'UTR were generated by the overlap extension PCR and then cloned into the psiCHECK-2 to establish a CTNNBIP1 3'UTR MUT plasmid.

To study the effect of androgen receptor (AR) on the expression of miR-423-3p, the coding sequence of the AR gene was cloned into the eukaryotic expression plasmid pCDNA3.1 basic by the endonuclease sites SalI and XhoI to established plasmid pCDNA-AR. All generated constructs were confirmed by sequencing. All of the constructs were confirmed by sequencing. All primer sequences are listed in Table S2.

Dual-luciferase reporter gene assay

In luciferase reporter assays, 293T cells were transfected in triplicate with luciferase vector and the indicated transcription factors using the transfection kit Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's instructions. The luciferase activity was measured using the dual-luciferase reporter assay (Promega, USA) at 48 h after transfection, the results were normalized by the activity of firefly luciferase against the activity of Renilla luciferase.

Hormone analysis

The culture medium and serum were collected and the T concentrations were measured. The measurements were performed by radioimmunoassay by the Institute of Beijing North Biotechnology.

Cell apoptosis analysis

Cell apoptosis was analyzed by the annexin V/propidium iodide (PI) staining. After stimulation, cells were washed twice with the ice-cold PBS solution and resuspended in 100 μL of 1× annexin binding buffer with 5 μL of annexin V-FITC (Beyotime Biotechnology, China) and 10 μL of PI (Beyotime Biotechnology, China). The cells were incubated for 15 min at room temperature in the dark and were then subjected to the flow cytometric analysis using FACSCalibur (Becton Dickinson, USA).

Cell cycle analysis

For cell cycle analysis, cells were harvested after treatment and then fixed in 70% ethanol at 4°C overnight, permeabilized, and stained with PI (Beyotime Biotechnology, China). After incubation for 30 min, 400 μL of binding buffer was added per sample, and the mixture was filtered through a 200-mesh nylon net prior to cell fluorescence measurement using FACSCalibur (Becton Dickinson, USA).

Western blotting

Cells were rinsed with the ice-cold PBS, and then the protein was extracted using the radioimmunoprecipitation assay method. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a

polyvinylidene fluoride (PVDF，Millipore, IPVH00010, USA) membrane as described previously [10, 11]. After washing, the membranes were incubated with one of the antibodies for: β-catenin (1:1000, 51067-2-AP, Proteintech), C-Myc (1:1000, 67447-1-Ig, Proteintech), CCND1 (1:1000, 26939-1-AP, Proteintech), β-actin (1:1000, 20536-1-AP, Proteintech), GAPDH (1:5000, 10494-1 Proteintech). Finally, the protein bands were visualized by the enhanced chemiluminescence kits (Beyotime Biotechnology, China).

miRNA database analysis

In this study, we selected miRNAs from two published articles (PMID: 25622783, 27001999) to identify those that are significantly differentially expressed in PCOS. We investigated small RNA sequencing (RNA-seq) data from two PCOS datasets and analyzed the differential expression of miRNAs using these databases. Through cross-analysis of the differentially expressed miRNAs identified in both datasets, we found seven miRNAs that were significantly differentially expressed.

Prediction of target genes

To identify potential target genes of miR-423-3p, we employed a bioinformatics approach using three online prediction tools: PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html), miRmap (http://mirmap.ezlab.org/), and TarBase (http://diana.imis.athena-innovation.gr/DianaTools/index.php). Each tool was used independently to predict potential binding sites for miR-423-3p within the 3' UTRs of candidate genes. The

predicted targets from all three databases were then intersected using an online Venn diagram tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) to identify common target genes. This intersection was visualized as a Venn diagram to highlight the overlapping predictions.

Statistical analysis

Data were analyzed using SPSS 16.0 software (SPSS Inc., USA) and GraphPad Prism 7 (GraphPad software, Inc., USA). The measurement data conforming to the normal distribution were expressed as mean±standard error of mean (SEM), in which the differences between the two groups were analyzed with the use of the t test and the differences among multiple groups were analyzed by one-way analysis of variance (ANOVA). The values of $*P<0.05$, $**P<0.01$, and $***P< 0.001$ are the indication of the statistical significance.

Results

The expression levels of miR-423-3p were significantly decreased in PCOS patients and PCOS-like mice.

By re-analysis of the data from the published databases [16, 17], we screened out seven differentially expressed miRNAs in hGCs of PCOS patients, and confirmed that miR-423-3p, the most significant changed miRNAs out of seven, was downregulated in hGCs from patients with PCOS (n=8) vs. healthy women (n=8) by using qRT-PCR analysis (Figure 1A).

To systematically investigate the relationship between miR-423-3p and PCOS, we analyzed the expression of miR-423-3p in a cohort of 40 PCOS patients and 30 age-matched non-PCOS women, which included the initial subset of 8 individuals (4 PCOS patients and 4 healthy controls) used for preliminary validation. The clinical characteristics of patients with PCOS and controls are listed in Table 1; it was observed that women suffering from PCOS exhibited a dramatic increase in the levels of T, LH, LH/ FSH, AMH, and BMI.

The expressions of miR-423-3p were significantly decreased in human serum (Figure 1B), FF (Figure 1C) and hGCs (Figure 1D) from PCOS patients compared with that of the healthy controls.

To better understand the clinical significance of miR-423-3p levels in PCOS, we performed the Pearson's rank correlation analysis between the serum miR-423-3p expression levels and the clinical features of 70 human samples. As shown in Table 2, a statistically significant negative correlations observed between miR-423-3p

311 expression and key clinical parameters, such as T, LH, LH/FSH ratio, AMH, and BMI,
312 suggested that a potential contribution of miR-423-3p to the dysregulation of endocrine
313 hormones and metabolic imbalances that is characteristic for PCOS pathogenesis.

314 To strengthen the robustness of our findings, we collected clinical samples from
315 another cohort in the Department of Reproductive Medicine, Jinling Hospital, Nanjing
316 University School of Medicine for independent verification. Consistent with our
317 original findings, miR-423-3p levels were significantly reduced in both serum and
318 hGCs of PCOS patients compared to control women (Figure Support 1). The
319 independent validation in a geographically distinct cohort strongly supports the
320 reliability of miR-423-3p as a biomarker and its involvement in PCOS pathogenesis.

321 To explore the possibility of miR-423-3p as a potential biomarker for PCOS, we
322 carried out the receiver-operating-characteristic (ROC) analysis of these human serum
323 samples. Our result showed that the area under the curve (AUC) was 0.82 (95%
324 confidence interval: 0.7158-0.9217; Figure 1E) for all patients, suggesting miR-423-3p
325 has a good serum diagnostic capacity to differentiate patients with PCOS from the
326 control women.

327 To further confirm the function role miR-423-3p in PCOS, we also established
328 several PCOS-like mouse models in this study. In consistent with the clinical results,
329 the expressions of miR-423-3p were significantly decreased in the ovaries of the
330 letrozole-induced PCOS mouse model (Figure 1F), the dehydroepiandrosterone
331 (DHEA)-induced PCOS mouse model (Figure 1G), and the dihydrotestosterone (DHT)-
332 induced PCOS mouse model (Figure 1H).

miR-423-3p promoted E_2 production and cell proliferation in GCs

To assess the potential involvement of miR-423-3p in GCs, the gain or loss of function experiments were carried out in KGN cell line and mouse granulosa cells (mGCs). The miR-423-3p mimic/inhibitor effectively increased/decreased the expression of miR-423-3p in KGN and mGCs (Figure S2A-S2D), respectively. In addition, we assessed the potential involvement of miR-423-3p in regulating the steroid hormone secretion and observed that overexpression of miR-423-3p increased the production of E_2 (Figure 2A), while knockdown of miR-423-3p decreased the production of E_2 in both KGN cells and mGCs (Figure 2B). Although CYP19A1 is a key enzyme in E_2 biosynthesis in the GCs steroidogenesis, we noted that miR-423-3p did not affect the expression of CYP19A1 (Figure 2C).

To determine whether miR-423-3p plays a role in cell growth or death, we first examined the apoptotic rate in the KGN by flow cytometry with Annexin V-FITC staining. As shown in Figure 2D, transfection of miR-423-3p mimic or inhibitor had no effect on the apoptosis rate of KGN cells.

Moreover, we noticed that miR-423-3p overexpression promoted the growth of KGN cells, while miR-423-3p knockdown significantly inhibited the growth of KGN cells (Figure 2E-2G). The results of the flow cytometry showed that miR-423-3p significantly decreased the ratio of cells in G0-G1 phase, and increased the percentage of cells in the S phase (Figure 2H). Taken together, these results showed that miR-423-3p promoted the proliferation of KGN by regulating the cellular processes.

***CTNNBIP1* is a potential target of miR-423-3p in KGN cells**

To investigate how the underlaying molecular mechanism of miR-423-3p regulates GCs proliferation, we predicted the target genes of miR-423-3p using the bioinformatics websites PITA, miRmap and TarBase. By intersecting the predictions from different databases, we obtained four candidate target genes of miR-423-3p (Figure 3A). The results of the RT-qPCR detection showed that overexpression of miR-423-3p significantly reduced the levels of *CTNNBIP1* (Figure 3B), suggesting that CTNNBIP1 may be an appropriate downstream target, which is involved in cell proliferation. Moreover, we found that miR-423-3p mimic decreased the relative luciferase activity of the CTNNBIP1 3'UTR-contained luciferase vector, compared with the mut vector control (Figure 3C-3D). The results of qRT-PCR showed that miR-423-3p mimic significantly reduced the expression of *CTNNBIP1*, while miR-423-3p inhibitor increased the expression of *CTNNBIP1* (Figure 3E).

We further detected the expressions of *CTNNBIP1* in hGCs of PCOS patients and ovaries of PCOS-like mice. The expressions of *CTNNBIP1* were significantly increased in the hGCs of PCOS patients and the ovaries of PCOS-like mice (Figure 3F-3I). These results indicated that miR-423-3p suppresses the expression of *CTNNBIP1* by directly binding to the domain of its 3′UTR.

miR-423-3p induced the proliferation of GCs through the WNT signaling pathway

Given that CTNNBIP1 is a type of β-catenin interacting protein, which can prevent the binding of β-catenin with TCF/LEF, thereby serving as a negative regulator of the activation of the WNT signaling pathway [18]. Therefore, we supposed that miR-423-3p promotes GCs proliferation by activating the WNT signaling pathway. To verify this

hypothesis, we detected the expression of the WNT signaling pathway-related proteins after overexpression or knockdown of miR-423-3p in KGN cells, respectively. The results showed that the miR-423-3p mimic promoted the expressions of β-catenin, C-Myc and CCND1 (Figure 3J and 3K), while the miR-423-3p inhibitor had the opposite effect (Figure 3L and 3M). Our data suggest that miR-423-3p may promote GCs proliferation, at least in part, by downregulating *CTNNBIP1* and subsequently modulating WNT signaling components.

DHT inhibited GCs proliferation via miR-423-3p.

Hyperandrogenemia is one of the typical characteristics of PCOS. By using the bioinformatics software JASPAR (https://jaspar.genereg.net/), we obtained several predicted androgen response elements (AREs) at the promoter region of miR-423-3p (Figure 4A). To confirm whether androgens regulate the promoter activity of miR-423-3p, we constructed and co-transfected the AREs overexpression plasmid pCDNA 3.1-AR (pAR) and the luciferase plasmid pGL3-miR-423-3p (pGL3-423) into 293T cells. The expressions of AR mRNA showed that the AR overexpression plasmid is constructed successfully (Figure 4B) by increasing the expression of AR in mRNA levels. And co-transfection of pGL3-423 and pAR significantly inhibited the luciferase activity of pGL3-423 (Figure 4C). DHT inhibited the luciferase activity of pGL3-423 in a dose-dependent manner (Figure 4D). Accordingly, qRT-PCR assays showed that DHT induction could repress the expression of miR-423-3p and promote the expression of *CTNNBIP1* in KGN in a dose-dependent manner (Figure 4E-4F). Furthermore, we demonstrated that DHT inhibited cell proliferation and induced G0-G1 phase arrest of

KGN cells, which could be partly reversed by over-expressed miR-423-3p (Figure 4G-4J).

Verifying the role of miR-423-3p in the progression of PCOS *in vivo*

To confirm whether miR-423-3p plays a critical role in the pathogenies of PCOS, we carried out a series of studies *in vivo*. Flutamide treatment meliorated the reproductive and metabolic phenotypes in the letrozole-induced PCOS mouse model [19]. In agreement, we found that flutamide treatment normalized the menstrual cyclicity (Figure 5A) and the circulating T levels (Figure 5B) in the letrozole-induced PCOS mice. Histologically, the increased cystic follicles were found in the ovaries of the letrozole-induced PCOS mice (Figures 5C and 5D). In addition, we observed that flutamide treatment significantly increased the expression levels of miR-423-3p up to normal levels and decreased the expression levels of *Ctnnbip1* in the ovaries of PCOS mice (Figure 5E and 5F).

Moreover, to evaluate the functional role of miR-423-3p in PCOS *in vivo*, we knocked down miR-423-3p by injecting the antagomir into the ovarian bursa of the WT mice. Antagomir at a dose of 200 pmol effectively inhibited the expression of miR-423-3p and increased the expression of *CTNNBIP1* until 15 days after injection (Figure 5G-5H). Thereafter, these conditions (200 pmol of antagomir and 15 days of treatment) were used for the subsequent investigations. Decreased expression of miR-423-3p and the increased expression of *CTNNBIP1* were found after the intraovarian injection of miR-423-3p antagomir in the ovaries of the WT mice (Figure 5J-5K). Thereafter, the dysregulated estrous cycle of mice injected with miR-423-3p antagomir was observed

(Figure 5L). Histopathological analysis showed the decreased numbers of corpora lutea and the thickness of the GC layer in the miR-423-3p antagomir group compared to those of the NC (Figure 5M-5O). These findings proved that downregulated miR-423-3p changed the ovary morphology of mice.

Overall, we demonstrated that miR-423-3p was regulated by androgen and played a crucial role in the pathogenesis of PCOS *in vivo* and *in vitro*.

Discussion

Increasing evidence indicates that miRNAs play crucial roles in PCOS, and their underlying mechanisms need to be further clarified. In this study, we determined that miR-423-3p was significantly downregulated in serum, FF, and hGCs of the PCOS patients, as well as in the ovaries of three types of induced PCOS-like mouse models. The ROC analysis demonstrated that miR-423-3p has advantages in the reliability of PCOS diagnosis. Overexpression of miR-423-3p promoted the proliferation of KGN via regulating the cellular processes by targeting *CTNNBIP1* which was upregulated in the GCs of PCOS patients. We demonstrated that miR-423-3p might be a novel mediator in the PCOS progress, which raises its potential as a new biomarker for PCOS diagnosis.

Among the participants recruited in the study, PCOS patients exhibited reproductive hormonal disturbances, such as elevated T, LH, and AMH, which have found similar outcomes in other studies [20, 21]. The results of Pearson's rank correlation coefficients analysis showed that the expression of miR-423-3p in serum was significantly negatively correlated with the contents of T, LH and AMH in serum, indicating that the imbalance of miR-423-3p in PCOS patients was significantly related to the reproductive hormone disorder in PCOS patients. Elucidating the function and mechanism of miR-423-3p in the disease process of PCOS is beneficial for understanding the pathogenesis of PCOS.

Several previous cancer studies have found that miR-423-3p could serve as a diagnostic marker for various cancers and participate in cancer progression by

promoting cell proliferation [22, 23]. Previous studies have shown that the expression of miR-423-5p was downregulated in GCs from patients with PCOS (n=46) compared to the controls (n=32) [24]. Moreover, they used an RNA oligonucleotide mixture containing miR-423-5p inhibitor, miR-33b mimic, and miR-142 mimic to study the effects on the cell proliferation of GCs, but did not analyze their function separately. miR-423-3p and miR-423-5p are two mature miRNAs respectively derived from the 3' and 5' ends of pre-miR-423. These "5p" and "3p" miRNAs exhibit biological differences in their stabilities and functions. In this study, we not only found that miR-423-3p was downregulated in GCs from patients with PCOS, but also significantly downregulated in FF and serum. And miR-423-3p overexpression promoted the growth of KGN cells, decreased the ratio of cells in the G0-G1 phase, and increased the percentage of cells in the S phase.

The observed regulation of E_2 production by miR-423-3p in GCs, despite no detectable changes in *CYP19A1* mRNA levels. This observation suggests that miR-423-3p may influence E_2 synthesis through alternative mechanisms that do not involve direct regulation of CYP19A1 expression. One possibility is that miR-423-3p affects the activity or post-translational modification of CYP19A1, rather than its expression levels. For instance, miR-423-3p might modulate the enzymatic activity of CYP19A1 by influencing its phosphorylation status or subcellular localization, thereby altering its efficiency in converting androstenedione to estradiol. This could explain the observed changes in E_2 production without corresponding alterations in *CYP19A1* mRNA or protein levels.Another potential explanation is that miR-423-3p impacts the expression

or activity of other enzymes or factors involved in the steroidogenic pathway. While CYP19A1 is a key enzyme in E_2 biosynthesis, the overall production of E_2 is also influenced by the availability of substrates, the activity of other steroidogenic enzymes such as StAR (steroidogenic acute regulatory protein), and the regulation of cholesterol transport into the mitochondria. miR-423-3p might indirectly affect E_2 synthesis by modulating these upstream processes or by regulating the expression of other genes that interact with CYP19A1 in the steroidogenic pathway. Additionally, post-translational modifications of CYP19A1 or interactions with miR-423-3p-regulated proteins (e.g., β-catenin) might modulate aromatase activity. This result also highlights the complexity of miRNA-mediated regulation in cellular processes. miRNAs can exert their effects through multiple targets and pathways, and the absence of a direct impact on CYP19A1 does not preclude indirect regulatory mechanisms. Future studies could investigate the potential targets of miR-423-3p that are involved in steroidogenesis, explore the post-translational modifications of CYP19A1 influenced by miR-423-3p, and examine the broader transcriptional and metabolic changes induced by miR-423-3p in granulosa cells to elucidate the precise mechanisms underlying its regulation of E_2 production.

Given that CTNNBIP1 is a type of β-catenin interacting protein, which can prevent the binding of β-catenin with TCF/LEF, thereby serving as a negative regulator of the WNT signaling pathway activation [17]. Previous studies have shown that high androgen treatment increased the *CTNNBIP1* expression in bovine GCs, and prevented CTNNB1 from interacting with the members of the WNT signaling pathway thereby inhibiting the cell cycle [25]. However, the molecular mechanism by which androgens

493 regulate the expression of *CTNNBIP1* has not been elucidated, nor has this regulatory
494 relationship been mentioned in humans. In this study, we confirmed that androgen also
495 increased the expression of *CTNNBIP1* in human KGN cells and revealed that hyper-
496 androgen inhibited the expression of miR-423-3p by binding to the AREs of the miR-
497 423-3p promoter region, thereby increasing the target gene *CTNNBIP1* in of miR-423-
498 3p and inhibiting KGN cell proliferation.

499 Hyperandrogenemia is not only a key character of PCOS, but also a leading cause
500 of subfertility or infertility. We found that the abundance of miR-423-3p in the serum
501 of women with PCOS is negatively related to the concentration of testosterone in the
502 serum. Our results indicated that a high concentration of androgen could downregulate
503 the expression of miR-423-3p, upregulate *CTNNBIP1*, and inhibit the proliferation of
504 KGN cells. The relationship between hyperandrogenemia and GCs proliferation arrest
505 has been reported several times [26, 27], and miR-423-3p might be a mediator or
506 treatment target in this pathway.

507 However, this study has some limitations: it only includes 40 PCOS patients and
508 30 healthy controls, investigating the level of miRNA candidates in a larger cohort is
509 needed to confirm their role for clinical use; the miRNA function was analyzed only *in*
510 *vitro*, and *in vivo* via the transient transfection with short duration, and the *in vivo*
511 studies using the transgenic or knockout mouse models are needed; We have proved
512 that downregulated miR-423-3p changed the ovary morphology of mice. Exploring
513 whether miR-423-3p mimic could be used as a therapeutic drug for PCOS is the
514 research focus of our ongoing study.

Conclusion

In summary, our findings showed that miR-423-3p was lower in the serum, FF, and GCs of women with PCOS, and its down-regulation was associated with hyperandrogenemia. miR-423-3p may promote the proliferation of GCs by targeting *CTNNBIP1*. Our study provides a new perspective molecular mechanism underlying the disordered folliculogenesis characteristic of PCOS and identifies miR-423-3p as a novel target for the treatment of PCOS.

Data Availability

Data is available from the author on reasonable request.

Acknowledgements

All authors have read the journal's policy on disclosure of potential conflicts of interest and declared no conflict of interest. All authors have read the journal's authorship agreement.

Author Contributions

SZ and XL designed the whole study; SZ, MW and YL performed the experiments; SZ, YL and DT prepared the figures; DT, SW and NR collected clinical samples from patients and completed testing; SZ, NA and XL wrote the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The datasets analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval and consent to participate

This study was approved by the Ethics Committee of the Medical University of Bialystok, and all samples were obtained with the informed consent from patients.

Consent for publication

All the authors approved for the publication of the manuscript.

Competing interests

None.

References

1. Jayasena CN, Franks S. The management of patients with polycystic ovary syndrome. *Nature reviews Endocrinology* 2014; **10**(10):624-636.
2. Lindholm A, Andersson L, Eliasson M, Bixo M, Sundström-Poromaa I. Prevalence of symptoms associated with polycystic ovary syndrome. *International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics* 2008; **102**(1):39-43.
3. Chi KN, Agarwal N, Bjartell A, Chung BH, Pereira de Santana Gomes AJ, Given R, Juárez Soto Á, Merseburger AS, Özgüroğlu M, Uemura H et al. Apalutamide for Metastatic, Castration-Sensitive Prostate Cancer. *The New England journal of medicine* 2019;**381**(1):13-24.
4. Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. *Reproduction (Cambridge, England)* 2001;**122**(6):829-838.
5. Das M, Djahanbakhch O, Hacihanefioglu B, Saridogan E, Ikram M, Ghali L, Raveendran M, Storey A. Granulosa cell survival and proliferation are altered in polycystic ovary syndrome. *The Journal of clinical endocrinology and metabolism* 2008; **93**(3):881-887.
6. Onalan G, Selam B, Baran Y, Cincik M, Onalan R, Gündüz U, Ural AU, Pabuccu R. Serum and follicular fluid levels of soluble Fas, soluble Fas ligand and apoptosis of luteinized granulosa cells in PCOS patients undergoing IVF. *Human reproduction (Oxford, England)* 2005; **20**(9):2391-2395.
7. Wang M, Sun J, Xu B, Chrusciel M, Gao J, Bazert M, Stelmaszewska J, Xu Y, Zhang H, Pawelczyk L et al. Functional Characterization of MicroRNA-27a-3p Expression in Human Polycystic Ovary Syndrome. *Endocrinology* 2018; **159**(1):297-309.
8. Hayes J, Peruzzi PP, Lawler S. MicroRNAs in cancer: biomarkers, functions and therapy. *Trends in molecular medicine* 2014; **20**(8):460-469.
9. Kalayinia S, Arjmand F, Maleki M, Malakootian M, Singh CP. MicroRNAs: roles in cardiovascular development and disease. *Cardiovascular pathology : the official journal of the Society for Cardiovascular Pathology* 2021; **50**:107296.
10. Zhang S, Liu Y, Wang M, Ponikwicka-Tyszko D, Ma W, Krentowska A, Kowalska I, Huhtaniemi I, Wolczynski S, Rahman NA et al. Role and mechanism of miR-335-5p in the pathogenesis and treatment of polycystic ovary syndrome. *Transl Res* 2023; **252**:64-78.
11. Liu Y, Zhang S, Chen L, Huang X, Wang M, Ponikwicka-Tyszko D, Rahman NA, Wolczynski S, Yao B, Li X. The molecular mechanism of miR-96-5p in the pathogenesis and treatment of polycystic ovary syndrome. *Transl Res* 2023; **256**:1-13.
12. Escobar-Morreale HF. Polycystic ovary syndrome: definition, aetiology, diagnosis and treatment. *Nature reviews Endocrinology* 2018; **14**(5):270-284.
13. Liu Y, Du SY, Ding M, Dou X, Zhang FF, Wu ZY, Qian SW, Zhang W, Tang QQ, Xu CJ. The BMP4-Smad signaling pathway regulates hyperandrogenism development in a female mouse model. *The Journal of biological chemistry* 2017; **292**(28):11740-11750.
14. Kafali H, Iriadam M, Ozardali I, Demir N. Letrozole-induced polycystic ovaries in the rat: a new model for cystic ovarian disease. *Archives of medical research* 2004; **35**(2):103-108.
15. Sullivan SD, Moenter SM. Prenatal androgens alter GABAergic drive to gonadotropin-releasing hormone neurons: implications for a common fertility disorder. *Proceedings of the National Academy of Sciences of the United States of America* 2004; **101**(18):7129-7134.

16. Xu B, Zhang YW, Tong XH, Liu YS. Characterization of microRNA profile in human cumulus granulosa cells: Identification of microRNAs that regulate Notch signaling and are associated with PCOS. *Molecular and cellular endocrinology* 2015; **404**:26-36.
17. Huang X, Liu C, Hao C, Tang Q, Liu R, Lin S, Zhang L, Yan W. Identification of altered microRNAs and mRNAs in the cumulus cells of PCOS patients: miRNA-509-3p promotes oestradiol secretion by targeting MAP3K8. *Reproduction (Cambridge, England)* 2016; **151**(6):643-655.
18. Stow JL. ICAT is a multipotent inhibitor of beta-catenin. Focus on "role for ICAT in beta-catenin-dependent nuclear signaling and cadherin functions". *American journal of physiology Cell physiology* 2004; **286**(4):C745-746.
19. Ryan GE, Malik S, Mellon PL. Antiandrogen Treatment Ameliorates Reproductive and Metabolic Phenotypes in the Letrozole-Induced Mouse Model of PCOS. *Endocrinology* 2018; 159(4):1734-1747.
20. Liu G, Liu S, Xing G, Wang F. 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* 2020; **20**:205-216.
21. Sir-Petermann T, Codner E, Maliqueo M, Echiburú B, Hitschfeld C, Crisosto N, Pérez-Bravo F, Recabarren SE, Cassorla F. Increased anti-Müllerian hormone serum concentrations in prepubertal daughters of women with polycystic ovary syndrome. *The Journal of clinical endocrinology and metabolism* 2006; **91**(8):3105-3109.
22. Liu Z, Cui Y. Bim's Effect on the Expression of miR-423-3p in Promoting Primary Hepatic Cancer (PHC) and Role of miR-423-3p in PHC Proliferation and Invasion. *Biochemical genetics* 2021; **59**(5):1247-1259.
23. Ma J, Huang W, Zhu C, Sun X, Zhang Q, Zhang L, Qi Q, Bai X, Feng Y, Wang C. miR-423-3p activates FAK signaling pathway to drive EMT process and tumor growth in lung adenocarcinoma through targeting CYBRD1. *Journal of clinical laboratory analysis* 2021; **35**(12):e24044.
24. Li Y, Xiang Y, Song Y, Wan L, Yu G, Tan L. Dysregulated miR-142, -33b and -423 in granulosa cells target TGFBR1 and SMAD7: a possible role in polycystic ovary syndrome. *Molecular human reproduction* 2019; **25**(10):638-646.
25. McFee RM, Romereim SM, Snider AP, Summers AF, Pohlmeier WE, Kurz SG, Cushman RA, Davis JS, Wood JR, Cupp AS. A high-androgen microenvironment inhibits granulosa cell proliferation and alters cell identity. *Molecular and cellular endocrinology* 2021; **531**:111288.
26. Chen MJ, Chou CH, Chen SU, Yang WS, Yang YS, Ho HN. The effect of androgens on ovarian follicle maturation: Dihydrotestosterone suppress FSH-stimulated granulosa cell proliferation by upregulating PPARγ-dependent PTEN expression. *Scientific reports* 2015; **5**:18319.
27. Pradeep PK, Li X, Peegel H, Menon KM. Dihydrotestosterone inhibits granulosa cell proliferation by decreasing the cyclin D2 mRNA expression and cell cycle arrest at G1 phase. *Endocrinology* 2002; **143**(8):2930-2935.

Figure 1. miR-423-3p expression levels significantly decreased in PCOS patients and in induced PCOS-like mice.

(A) qRT-PCR was used to screen the differentially expressed miRNAs in hGCs of the PCOS patients (n=8) and healthy women (n=8). (B) qRT-PCR detection of miR-423-3p expression in serum from 40 PCOS patients and 30 normal control. (C) miR-423-3p expression in FF of PCOS patients detected by qRT-PCR. (D) miR-423-3p expression in hCCs of PCOS patients detected by qRT-PCR. (E) ROC analysis of the serum miR-423-3p for discriminating PCOS. miR-423-3p expression in ovaries of the letrozole-induced mice (n=10/group) (F), DHEA-induced mice (n=8/group) (G) and DHT mice (n=8/group) (H) detected by qRT-PCR. Data are mean±SEM and were analyzed by Student's t-test. * P <0.05, ** P <0.01, *** P <0.001.

Figure 2. miR-423-3p promoted E_2 production and GCs proliferation in KGN

(A-B) E_2 accumulation were measured via radioimmunoassay 48 h after transfection with miR-423-3p mimic/inhibitor and NC in KGN and mGCs. (C) The mRNA expression levels of *CYP19A1* in KGN and mGCs were measured by qRT-PCR. (D) Effects of miR-423-3p on cell apoptosis using flow cytometry in KGN 48 h after treatment. (E-F) The proliferation of non-transfected or transfected KGN cells was measured using CCK8 after 24–72 h. (G) Using Ki67 assay, the percentage of proliferating cells (green/DAPI) differed between cells transfected with miR-423-3p mimic/inhibitor (48 h post-transfection). Scale bar: 50 μm. (H) The effect on cell cycle was assessed by PI staining in KGN (48 h post-transfection). Data are mean±SEM and were analyzed by Student's t-test.. * P <0.05, ** P <0.01, *** P <0.001.

Figure 3. CTNNBIP1 is a potential target of miR-423-3p.

(A) Four candidate target genes for miR-423-3p were selected based on the intersection of the predicted results from three databases. (B) The mRNA expression levels of four candidate target genes in KGN after transfection with miR-423-3p mimic. (C) miR-423-3p specific binding sites on CTNNBIP1 predicted by TarBase. (D) The regulative relationship between CTNNBIP1 and miR-423-3p was assessed in 293T cells by a dual-luciferase reporter gene assay. (E) The mRNA expression levels of *CTNNBIP1* in KGN after transfection with miR-423-3p mimic and inhibitor. (F) The mRNA expression levels of *CTNNBIP1* in hGCs of PCOS patients (n = 40) and normal controls (n = 30) by qRT-PCR. The mRNA expression levels of *CTNNBIP1* in ovaries of letrozole-induced PCOS-like mice (G), DHEA-induced PCOS-like mice (H), DHT PCOS-like mice (I). (J and K) The protein levels of β-catenin、C-Myc、CCND1 and β-actin in KGN after transfection with miR-423-3p mimic for 48 h. (L and M) The protein levels of β-catenin、C-Myc、CCND1 and β-actin in KGN after transfection with miR-423-3p inhibitor for 48 h. Data are mean±SEM and were analyzed by Student's t-test. * P <0.05, ** P <0.01, *** P <0.001.

Figure 4. DHT inhibited GCs proliferation via miR-423-3p.

(A) The predicted binding sites of ARE by JASPAR software in the 2000 nucleotides upstream of the miR-423-3p. (B) The efficiency of AR overexpression vector (pAR) was detected in KGN. (C) The 293T cells were transfected with pGL3-423 and pAR and then subjected to luciferase measurement 48 h after transfection. (D) The 293T cells were transfected with pGL3-423 and treated with DHT and DMSO as a control. At 36

h after transfection, luciferase activity was measured.The expression of miR-423-3p (E) and *CTNNBIP1* (F) in KGN cells with different does of DHT. (G-H) Cell proliferation of KGN cells in each group was detected by Ki67 assay. Scale bar: 50 μm. (I) Cell proliferation of KGN cells in each group was detected by CCK-8 assay. (J) Cell cycle distribution of KGN cells in each group was detected by flow cytometry. Data are mean±SEM and were analyzed by one-way ANOVA. * P <0.05, ** P <0.01, *** P <0.001.

Figure 5. Verifying the role of miR-423-3p in the progression of PCOS *in vivo*.

(A) Estrous cycle was assessed by vaginal cytology for ten consecutive days. P, proestrus; E, estrus; M, metestrus; D, diestrus. (B) Serum T of mice was measured by radioimmunoassay (n=5/group). (C) HE staining showed morphology of ovarian tissues (n=5/group). (D) Number of antral follicles were counted after HE staining (n=5/group). The mRNA expression levels of miR-423-3p (E) and *CTNNBIP1* (F) in the ovaries of mice (n=5/group). (G and H) The expression of miR-423-3p and *CTNNBIP1* were determined by qRT-PCR 24 h after ovary- intrabursal injection of different dose of miR-423-3p antagomir (n=4/group). (I) The expression levels of miR-423-3p were detected at time course after 200 pmol antagomir injection (n=4/group). (J) The expression of miR-423-3p determined by qRT-PCR 15 days after ovary- intrabursal injection of antagomir (n=5/group). (K) The expression of *CTNNBIP1* determined by qRT-PCR 15 days after ovary- intrabursal injection of antagomir (n=5/group). (L) Estrous cycle after ovary-subcutaneous injection of miR-423-3p antagomir (n=5/group). (M) Morphological analysis of the ovaries from WT mice injecting the miR-423-3p

antagomir and NC by HE staining (n=5/group). Scale bars:100 μm. Number of corpus luteum (N) and thickness of granulosa cell layer (O) were measured after HE staining (n=5/group). Data are mean±SEM and were analyzed by one-way ANOVA. * P <0.05, ** P <0.01, *** P <0.001.

Figure 6. Graphical abstract

Here, we found a novel, potent diagnostic biomarker of PCOS, circulating miR-423-3p, which was related to abnormal patients' characteristics. miR-423-3p regulated the proliferation of KGN cells by directing targeting CTNNBIP1 to regulate the WNT signaling pathway. Importantly, ovarian intrabursal injections of miR-423-3p antagomir induced PCOS-like reproductive phenotype in WT mice. Figure done by Biorender.com.