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Functional roles of miR-27a-3p in human PCOS

Functional characterization of microRNA-27a-3p expression in human polycystic ovary syndrome

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Abbreviations :

miR-27a-3p, microRNA-27a-3p; miR-NC, miRNA negative control; E_2, estradiol; GCs, granulosa cells; P_4, progesterone; PI, propidium iodide; qPCR, real-time quantitative PCR; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PCNA proliferating cell nuclear antigen; PCOS, polycystic ovary syndrome; SMAD5, sterile alpha motif domain containing; 3'UTR, 3' untranslated regions; STAT1, signal transducer and activator of transcription 1; STAT3, signal transducer and activator of transcription 3.

The goal of this study was to characterize the function of microRNA-27a-3p (miR-27a-3p) in polycystic ovary syndrome (PCOS). MiR-27a-3p expression was analyzed in excised granulosa cells (GCs) from 21 PCOS and 12 normal patients undergoing IVF cycle treatments, and in 17 non-treated cuneiform ovarian resection PCOS samples and 13 control ovarian samples-from non-PCOS patients. We found that the expression of miR-27a-3p was significantly increased in

both excised GCs and the ovaries of PCOS patients compared to the controls. Insulin treatment of the human granulosa-like tumor cell line KGN resulted in decreased downregulated the expression of miR-27a-3p, and this effect appeared to be mediated by STAT1 and STAT3. The overexpression of miR-27a-3p in KGN cells inhibited SMAD5, which in turn decreased cell proliferation and promoted cell apoptosis. After KGN cells were stimulated with insulin for 48 h, there was increased expression of SMAD5 protein and decreased apoptosis. Additionally, knockdown/overexpression of *SMAD5* in KGN cells reduced/increased cell number and promoted/ inhibited cell apoptosis. Insulin-stimulated primary GCs isolated from PCOS patients, in contrast to normal GCs or KGN cells, did not exhibit decreased miR-27a-3p expression. The differences in the expression levels in KGN cells and human PCOS GCs are likely explained by increased miR-27a-3p expression in the GCs caused by insulin resistance in PCOS. Taken together, our data provided evidence for a functional role of miR-27a-3p in the GCs dysfunction that occurs in human PCOS patients.

A functional role was identified for the upregulated miR-27a-3p expression in the observed granulosa cell dysfunction in human PCOS patients.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is one of the most common endocrine and metabolic disorders in women of reproductive age, and is characterized by hyperandrogenemia with arrest of follicle growth and ovulatory dysfunction (1-3). It has been reported that 75 % of infertile women may suffer from PCOS worldwide (4). The majority of PCOS patients exhibit insulin resistance (IR), leading to hyperinsulinemia and increased risk for type 2 diabetes mellitus and cardiovascular disease (5). PCOS patients often have elevated levels of ovarian androgens, and about 50 % of patients show elevated serum dehidroepiandorstendione sufate (DHEAS), potentially of adrenal origin (6). Several genetic and environmental factors have been reported to contribute to the development of PCOS (2,7). However, the underlying molecular mechanism leading to PCOS remains unclear.

Granulosa cells (GCs) play an important role during folliculogenesis. Proliferating GCs provide a suitable microenvironment for follicle growth initiation, oocyte maturation, and atresia (8). Dysfunction of GCs in PCOS patients including decreased proliferation, increased apoptosis, or unbalanced hormone production may contribute to abnormal folliculogenesis (9,10).

MicroRNAs (miRNAs) are 21-25-nucleotide non-coding RNAs that are able to post-transcriptionally regulate gene expression (11). These miRNAs function as important regulators of various physiological and pathological processes, such as development, cell proliferation, differentiation, migration, and apoptosis (12-14). It has been suggested that ovarian miRNAs may play a role in PCOS (15,16). McCallie et al. showed that the expression levels of 6 miRNAs including hsa-let-7a, hsa-miR-19a, hsa-miR-19b, hsa-miR-24, hsa-miR-92, and hsa-miR-93, were noticeably downregulated in PCOS blastocysts compared to controls (17). Similarly, Roth et al. reported decreased expression of miR-132 and miR-320 in the follicular fluid of PCOS

patients (18). In addition, several serum miRNAs differentially expressed in PCOS patients were suggested to play key roles in pathological insulin signaling, inflammation, and hormone secretion (15). Although the abnormal expression of miRNAs directly affects follical development and the normal physiological function of ovaries, the role of miRNAs in PCOS GCs remains unknown.

Our recent profiling data analysis predicted that novel miRNAs may be involved in the pathophysiology of PCOS (3). The recent advancements in high-throughput profiling and sequencing provide a comprehensive tool to understand the various signaling pathways and molecular networks involved in the etiology and pathophysiology of PCOS. Thus, our next goal was to dissect the functional role of individual miRNA participating in the pathology. In this study, we focused on the functional characterization of miR-27a-3p expression in ovaries and GCs from PCOS patients. Additionally, we analyzed miR-27a-3p function using a human granulosa tumor-like cell line to explore the mechanistic role of miR-27a-3p in the dysfunction of GCs.

MATERIALS AND METHODS

Human granulosa cells (GCs)

We analyzed GCs excised from a total of 42 donor patients [healthy control patients, n=17 (qPCR analysis, n=13; *in vitro* n=4) and PCOS patients, n=25 (qPCR analysis, n=21; *in vitro* n=4)] undergoing in vitro fertilization (IVF) stimulation cycles with the classical standard protocol of GnRH antagonist (Cetrotide) 0.25 mg subcutaneously. (s.c.) + recombinant FSH 150 IU s.c. + ovulation peak induced by 0.2 mg (Gonapeptyl) daily ferring s.c. GnRH agonist (19). The revised criteria from the Rotterdam European Society of Human Reproduction (ESHRE) and Embryology/American Society (ASRM) (17,20) were used for diagnosis of PCOS. GCs were obtained by follicular fluid aspiration. After oocyte removal, follicular fluid containing GCs were pooled and centrifuged at 250 x *g* for 10 min. The fraction containing the GCs was carefully aspirated, transferred to a new centrifuge tube, resuspended, washed in PBS (Gibco, USA), and then centrifuged at 250 x *g* for 5 min. GC pellets were lysed with TRIsure (Bioline, UK) for total RNA extraction or resuspended in culture medium for *in vitro* studies. Only GCs from follicules with mature oocytes (MII) were included in this study. The local Human Investigation Ethics Committee at the Medical University of Bialystok, Bialystok, Poland approved the study (license number R -I - 002 /146/2016). Written informed consent was obtained from all patients/donors before the study.

Human ovarian samples

We examined ovaries from a total number of 30 patients (normal healthy control patients, n=13 and PCOS patients, n=17). This study group included patients of reproductive age, with PCOS diagnosed according to the ESHRE and ASRM criteria (20). The PCOS patients qualified for an ovarian cuneiform resection by laparoscopy due to a lack of ovarian response to multiple (3-6) cycles of clomiphene citrate stimulation or treatment with pituitary gonadotropin hormones. The

control ovarian group (n=13) consisted of patients of reproductive age who did not meet the PCOS diagnostic criteria (20) but who had undergone surgical procedures for other reasons, such as small ovarian cysts, fibroids, chronic pelvic pain, or for diagnostic infertility laparoscopy that may have required a biopsy of healthy tissues or lesion removal with a healthy tissue margin. The patient exclusion criteria for both groups were as follows: ovarian, adrenal, endometrial, cervical and breast neoplasms, pelvic endometriosis, congenital adrenal hyperplasia, clinically and/or laboratory-confirmed endocrine disease (thyroid dysfunction, acromegaly, gigantism, or Cushing's disease), diabetes type I or II, hyperprolactinemia, and unexplained vaginal bleeding. Ovarian cuneiform resection samples were collected during laparoscopic surgery, protected against RNA degradation by mixing with RNA later (Life Technologies, USA), snap-frozen in liquid nitrogen, and stored at -80 °C until analyzed. The local Human Investigation Ethics Committee at the Medical University of Poznan, Poznan, Poland approved the study (license number 955/16). Written informed consent was obtained from all patients/donors before the study.

Total RNA isolation

Total RNA was isolated from human ovaries and GCs using a Trizol-based standard extraction protocol according to the manufacturer's instructions (Life Technologies, USA). The total RNA concentration was quantified using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, USA).

Reverse transcription (RT) and real-time quantitative PCR (qPCR)

For normal and PCOS ovarian/GCs total RNA samples, RT was performed with the SensiFAST™ cDNA Synthesis Kit (Bioline, UK). Prior to cDNA synthesis, 1μg of total RNA was incubated with 1 μl of DNase I according to the manufacturer's protocol (Invitrogen, USA). RT was performed in a thermal cycler using the following conditions: 25 °C - 10 min, 42 °C - 15 min, 48 °C - 15 min (an additional step for highly-structured RNA), 85 °C - 5 min, and then 4 °C. Next, qPCR was performed with DyNAmo HS SYBR Green qPCR Kit (Thermo Fisher Scientific, USA) and the primers indicated in Table 3 using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). Following an initial denaturation at 95 °C for 10 min, 40 cycles of PCR amplification were performed of 95 °C for 10 s, 58-60 °C for 20 s, and 72 °C for 15 s.

For KGN and Hela cells, RT was performed with the cDNA Synthesis Kit (Thermo Scientific, USA) using the following conditions: 42°C-90 min, 95 °C - 5 min, followed by a hold at 4 °C. Next, cDNA was amplified in a Roche LightCycler 480 system (Roche, Switzerland), using SYBR Green Master Mix Reagent (Roche, Switzerland). Cycling conditions were as follows: denaturation for 10 min at 95 °C followed by 40 cycles of 10 s at 95 °C, 10 s at 60 °C, and 10 s at 72 °C.

The specificity of qPCR amplification was verified by performing a melting curve analysis and agarose gel electrophoresis. Relative gene expression was calculated by the efficiency-corrected ΔΔCt method, normalized to the index (geometric mean) of four housekeeping genes:

cyclophilin A (PPIA), β-actin (ACTB), β-glucuronidase (GUSB), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Each sample in every group was measured in duplicate.

RT and qPCR to measure miRNA expression

RT was performed with the TaqMan® MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol in a thermal cycler using the following conditions: 16 °C - 30 min, 42 °C - 30 min, 85 °C - 5 min, and then 4 °C. The miRNA primers/probes used in this study were purchased commercially (Thermo Fisher Scientific, USA) and are shown in Supplemental Table 3. For the PCOS samples, qPCR was performed with TaqMan® Universal Master Mix II (Thermo Fisher Scientific, USA) using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). Following an initial denaturation at 95 °C for 10 min, 40 cycles of PCR amplification were performed at 95 °C for 15 s and 60 °C for 1 min. For KGN and Hela cells, qPCR was performed in a Roche LightCycler 480 system (Roche, Switzerland), using SYBR Green Master Mix Reagent (Roche, Switzerland) with the following conditions: denaturation for 10 min at 95 °C followed by 40 cycles of 10 s at 95 °C, 10 s at 60 °C, and 10 s at 72 °C. Relative miRNA expression was calculated by the efficiency-corrected ΔΔCt method, and was normalized to the endogenous control U6 snRNA. Each sample in every group was measured in duplicate.

Cell culture

For i*n vitro* studies, we used human primary GCs from PCOS and normal healthy female patents, as well as a steroidogenic human granulosa-like tumor cell line KGN (21). GCs were obtained from woman with PCOS (n=4) and healthy controls (n=4) undergoing *in vitro* fertilization treatment. After oocyte removal, samples of follicular fluid containing GCs were sedimented for 30 min. The fraction with GCs was carefully transferred to a new tube and resuspended in culture medium [DMEM/F12 medium (Sigma, USA) with 10 % FBS (Gibco, USA) and 1 % antibiotics (100 U/ mL penicillin and 100 µg/mL streptomycin; Sigma, USA). KGN cells are undifferentiated and maintain the physiological characteristics of ovarian cells, with normal expression of the functional FSH receptor and CYP19A1 (21). KGN cells were grown in DMEM/ F12 (Sigma, USA) supplemented with 10 % fetal bovine serum (Gibco, USA) and 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin; Sigma, USA), and cultured at 37°C under 5% CO_2. HeLa cells (ATCC CCL-2) were grown in DMEM/ High glucose (Sigma, USA) supplemented with 10% fetal bovine serum (Gibco, USA), and grown under the same culture conditions described above.

Cells were equally distributed onto a 12- well plate. After 12 h of incubation with full medium, cells were rinsed with PBS (Gibco, USA) and serum-starved for 12 h [DMEM/F12 medium with 0.5 % charcoal-stripped FBS (Gibco, USA)]. Next, the medium was exchanged for fresh medium with stimulation of 100, 10, or 1 ng/ml insulin, and full medium was used as a positive control. Cells were treated for 24 h, rinsed with PBS and then collected with the TRI Reagent™ Solution (Thermo Scientific, USA).

KGN cells or primary GCs from PCOS/ normal healthy control patients were plated in 6-well plates at 2×10^5 per well. After starving for 12 h, the cells were treated without (0) or with insulin (Sigma, USA) at concentrations of 1, 10, and 100 ng/mL for 24 h to test the expression of miR-27a-3p (for both primary GCs and KGN cells) or 48 h to test the protein expression level of SMAD5 (for KGN cells).

Plasmids

To experimentally determine if miR-27a-3p targets *SMAD5*, both the wild-type (5'-acuuugaguacagauacugugag-3') and mutated (5'-acuuugaguacagaugacgcaag-3') 3'-UTRs of the *SMAD5* gene were cloned into the psi-CHECK-2 dual luciferase reporter vector (Promega, USA) using Not I and XhoI restriction sites. To study the effect of STAT1 and STAT3 on the expression of miR-27a-3p, the coding sequence of the STAT1 gene was cloned into the eukaryotic expression plasmid pEGFP-C1. To assess the effect of SMAD5 overexpression on KGN cells, we cloned the coding sequence of *SMAD5* into the eukaryotic expression plasmid pCDNA3.1. All generated constructs were confirmed by sequencing.

Transient transfection and luciferase activity assay

KGN and/or HeLa cells were transfected with miRNA mimics or a miRNA negative control (miR-NC) using Lipofectamine 3000 (Invitrogen, USA) following the manufacturer's protocol. miRNA mimics and miR-NC were chemically synthesized by Shanghai GenePharma (Shanghai, China). Briefly, the day before transfection, cells were plated with normal growth medium and were 70-80% confluent at the time of transfection. The time of the start of transfection was considered time 0.

For reporter assays, Hela cells were transiently transfected with reporter constructs together with miRNA mimics and miR-NC. Cell extracts were prepared 48 h after transfection, and the ratio of Renilla/firefly luciferase was measured with the Dual-Luciferase Reporter Assay System (Promega, USA).

Cell proliferation

Cell proliferation was measured with the Cell Counting Kit-8 (CCK-8, Beyotime Institute of Biotechnology, China). Cells were seeded into 96-well plates at 1×10^4 cells per well and cultured in growth medium. After stimulation, 10 μL of the CCK-8 solution was added to each well and then incubated for 2 h. The cell numbers were measured in a PowerWave HT Microplate Spectrophotometer (BioTek, USA) by measuring the absorbance at a wavelength of 450 nm (OD 450).

Hormone analysis

The culture medium was collected and the estradiol (E_2) and progesterone (P_4) concentrations were measured. The measurements were performed by radioimmunoassay by the Institute of Beijing North Biotechnology. Both the inter- and intra- variation of the E_2 and P_4 assays were below 10 %.

Cell apoptosis analysis

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

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. After incubation for 15 min, 400 μL 1× of binding buffer was added per sample and the mixture was filtered through a 200-mesh nylon net prior to cell fluorescence measurment using FACSCalibur (Becton Dickinson, USA). Data were analyzed by Flowjo software.

Western blotting

Cells were rinsed with ice-cold PBS and then the protein was extracted using the RIPA method. Protein samples were separated by SDS-PAGE and then transferred to a PVDF membrane as described previously (22). After washing, the membranes were incubated with antibodies. Finally, the films were scanned and quantified using ImageJ software. Antibodies used in this study are shown in Table S2.

Microarray data

The expression of SMAD5 in PCOS GCs was mined from the public data set GSE34526 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE34526).

Statistical analysis

The results are presented as the means ± standard error of the mean (SEM). Statistical significance was assessed by the two-tailed Student's t test using the GraphPad Prism 5 software. A P-value < 0.05 was considered statistically significant.

RESULTS

Changes in miR-27a-3p expression in PCOS ovaries and GCs.

Using data from the published database (3), of the 59 known miRNAs differentially expressed in PCOS *vs.* control GCs (fold change > 2 and p < 0.05), 21 miRNAs were upregulated and 38 were downregulated in PCOS GCs. We found that the up-regulated miRNAs miR-27a-3p and miR-27a-5p correlated with increased serum testosterone (T) in GCs from PCOS (Supplemental Table 1 [Table S1]). To validate the miRNA microarray results, we performed real time quantitative real-time PCR (qPCR) analysis on samples from non-treated healthy controls (n=13) and PCOS patients (n=17). Interestingly, miR-27a-3p was significantly upregulated in both GCs and ovaries from PCOS patients, but no statistically significant changes were observed for miR-27a-5p (Figures 1A-D). MiR-27a-3p is located on chromosome 8 in mouse and chromosome 19 in human, and is proximal to two other miRNAs, miR-23a and miR-24-2. We also checked the expression of these

two miRNAs, but no significant changes in miR-24-2 and miR-23a expression were observed in PCOS GCs and ovaries compared to the level of expression in the controls (Figures 1E-H). The hormonal treatment in the IVF protocol used for both the control and PCOS patients from whom the GCs were excised likely contributed to the observed identical miRNA expression patterns of the non-treated PCOS and control ovary miRNAs (Figures 1A-H). The characteristics of the participants are presented in Table 1.

Insulin induced the down-regulation of miR-27a-3p expression mediated by STAT1 and STAT3 in human granulosa-like tumor KGN cells.

Hyperinsulinemia is one of the most common features of PCOS. We hypothesized that miR-27a-3p may be regulated by insulin. We performed qPCR using KGN cells (21) stimulated with insulin to assess the expression of miR-27a-3p. The qPCR results showed approximately 0.40-fold and 0.50-fold decreases in the levels of miR-27a-3p after 24 h treatment with 10 and 100 ng/mL insulin respectively (Figure 2A). To determine the underlying mechanism of how insulin led to the downregulation of miR-27a-3p, we analyzed the region of 2000 nucleotides upstream of miR-27a-3p using the JASPAR database. Among the potential binding transcription factors (TFs), two TFs **(**signal transducer and activator of transcription 1/3 [STAT1/3] **)** were selected based on their multiple conserved predicted binding sites and their known roles in proliferation and apoptosis (Figure 2B). To clarify the effects of the two TFs, STAT1 and STAT3, on the expression of miR-27a-3p, we transfected a STAT1-overexpression vector (pEGFP-STAT1) and a control vector, pEGFP into KGN cells. The qPCR results showed a significant decrease of miR-27a-3p in the STAT1-overexpressed cells compared with controls (Figure 2C). To explore whether these two TFs are regulated by insulin, we measured the protein levels in KGN cells treated with insulin for 48 h and found that STAT1 and STAT3 were obviously decreased in the insulin-treated cells, and that this effect was dose-independent (Figures 2D-F). These data demonstrated that miR-27a-3p could be regulated by insulin and that this effect may be mediated by STAT1 and STAT3.

Overexpression of miR-27a-3p decreased GC proliferation and promoted apoptosis in KGN cells.

In order to assess the functional role of miR-27a-3p in GCs, we transfected either miR-27a-3p mimics or miR-NC into the KGN cell line. The expression of miR-27a-3p in cells after transfection was measured by qPCR analysis. Transfection with miR-27a-3p mimics increased intracellular miR-27a-3p levels by approximately 1700-fold (Figure 3A). Recent studies have reported that differential expression of miRNAs is involved in hormone secretion and proliferation in GCs (23-25). Next, we studied the production of (17) estradiol (E_2) and progesterone (P_4) by KGN cells in response to the overexpression of miR-27a-3p. The overexpression of miR-27a-3p had no effect on E_2 and P_4 production in GCs (Supplementary Figure 1A [Figure S1A]). Using the CCK-8 assay, we observed a significantly reduced number of GCs 48 h after transfection of miR-27a-3p mimics into GCs (P<0.05) (Figure 3B). To further demonstrate how miR-27a-3p affects GCs growth, we examined the apoptotic rate and cell cycle in KGN. Using Annexin V-FITC and propidium iodide (PI) staining and flow cytometry 48 h

post-transfection, we observed that the overexpression of miR-27a-3p significantly increased the apoptotic rate of KGN cells (P<0.05) (Figures 3C, S1B). Using PI staining and flow cytometry analysis, we observed no changes in cell numbers of G0/G1 phase and G2/M phase (Figure 3D). Also, we found that the protein expression levels of PCNA and the mRNA expression level of cell cycle-related genes (CDK6, cyclin D1, and cyclin E) were not altered (Figures 3E and 3F). All of these data indicated that miR-27a-3p decreased the number of GCs by promoting cell apoptosis.

SMAD5 is a potential target of miR-27a-3p in KGN cells.

To study the effects of miR-27a-3p on apoptotic target mRNAs, seed-sequence-based predictions were performed in miRDB and TargetScan. Among the candidate targets of miR-27a-3p, we found that the SMAD5 gene seemed an appropriate candidate, because it is involved in cell apoptosis (26). The 3'-UTR of SMAD5 mRNA contains an evolutionarily conserved miR-27a-3p putative binding site in vertebrates (Figure S1C). To determine whether SMAD5 is a true target of miR-27a-3p, we constructed dual luciferase reporter vectors containing the wild-type 3'-UTR of SMAD5, as well as a mutant form of the miR-27a-3p seed sites (Figure S1D). Hela cells were co-transfected with the reporter plasmids and miR-27a-3p mimics or miR-NC, and harvested 48 h after transfection and assayed for dual luciferase activity. As shown in Figure 3G, the luciferase activity from the wild-type SMAD5 3'-UTR was reduced by 40% in the presence of the miR-27a-3p mimics. However, the mutant form of the seeding site abolished the silencing effects of miR-27a-3p on SMAD5 3'-UTR reporter activity. These results indicated that miR-27a-3p suppresses SMAD5 expression by directly binding to its 3'-UTR.

To validate that the SMAD5 gene is a target of miR-27a-3p, KGN cells were transfected with miR-27a-3p mimics or miR-NC. Next, qPCR and Western blot analysis were performed and the results revealed that the expression of SMAD5 mRNA (Figure 3H) and protein (Figure 3I) in KGN cells was significantly lower in miR-27a-3p transfected KGN compared to the control cells. Thus, our results demonstrated that the SMAD5 gene is a miR-27a-3p target and that miR-27a-3p regulates SMAD5 gene expression both at the transcriptional level and the post-transcriptional level. Additionally, we measured SMAD5 mRNA expression in the PCOS GCs and ovarian samples, and observed that SMAD5 was significantly downregulated in the PCOS group (Figures 3J and 3K). Interestingly, the expression profiling of human miRNAs from the public database (27) showed a similar result (Figure 3L). Taken together, the *SMAD5* gene seemed a likely potential target of miR-27a-3p.

Knockdown/overexpression of SMAD5 reduced/increased apoptosis in KGN cells.

We knocked down *SMAD5* expression by using siRNA (si-SMAD5). The expression of *SMAD5* mRNA (Figure 4A) and protein (Figure 4B) was decreased in cells transfected with si-SMAD5 for 48 h compared to the expression levels of the controls. Next, we measured the production of E_2 and P_4 by KGN cells, and found that knockdown of SMAD5 had no effect on E_2 and P_4 production in KGN cells (Figures S2A and S2B). By using the CCK-8 assay, we observed a significantly reduced number of KGN cells 24 h after transfection with si-SMAD5 (P<0.05)

(Figure 4C). Using Annexin V-FITC and PI staining and flow cytometry 48 h post-transfection, we observed that knockdown of SMAD5 significantly increased the apoptotic rate of KGN cells (P<0.05) (Figures 4D and S2D). Using PI staining and flow cytometry analysis, we observed no changes in the cell numbers of G0/G1 and G2/M phases (Figure S2C). Conversely, when we overexpressed SMAD5 in KGN cells, we observed an increased cell number (Figures 4G and S2H) and reduced apoptosis rate (Figures 4H and S2I) but no obvious change in the cell cycle (Figure S2G) or the hormone secretion of E_2 and P_4 (Figures S2E and S2F) (Figures 4E and 4F). All of these data showed that *SMAD5* knockdown decreased the number of KGN cells by promoting cell apoptosis, which is similar to the effects of miR-27a-3p overexpression.

Insulin induced the upregulation of SMAD5 protein level and reduced apoptosis in KGN cells

We showed that miR-27a-3p was be regulated by insulin and promoted apoptosis in KGN cells. There are several reports that insulin could promote KGN cell proliferation (28,29). Therefore, we next explored the proliferation and apoptotic effects of insulin on KGN cells. We applied an insulin concentration of 100 ng/mL for 48 h and observed a significantly increased number of GCs by CCK-8 assay (P<0.05), (Figure 5A) and inhibited apoptosis in KGN cells (Figures 5B and S3A). Additionally, the expression of SMAD5 was increased after the insulin treatment (Figures 5C and 5D). These data further demonstrated that miR-27a-3p promoted apoptosis in KGN cells by targeting SMAD5.

Insulin treatment of GCs derived from PCOS and normal patients

GCs from PCOS and normal healthy patients were stimulated with varying doses of insulin to assess the effects on the expression of miR-27a-3p. QPCR analysis showed no significant changes in the PCOS GCs after insulin treatment *vs.* non-treated PCOS GCs, but miR-27a-3p levels were higher compared to the GCs from normal healthy women (Figure 6).

DISCUSSION

A growing number of studies suggest that miR-27a-3p plays important functions in various physiological and pathological processes (30-32). Zhou et al. found that miR-27a-3p targets BTG2 in gastric cancer, and plays a role as a growth-promoting and anti-apoptotic factor (33). Another study reported that miR-27a-3p can induce apoptosis in human prostate cancer cells (34). These pro- or anti-apoptotic properties of miR-27a-3p suggested that miR-27a-3p may play different functions in different physiological states and pathological conditions according to the environmental context. GC apoptosis can induce follicular atresia, which leads to oocyte maturation and follicular developmental disorder. A high rate of apoptosis in GCs can prevent the conversion of androgen to estrogen, causing local hormones changes within the follicle and disturbing normal follicular development (35,36). Our data showed that the level of miR-27a-3p was increased/ upregulated in GCs of PCOS patients and that overexpression of miR-27a-3p in KGN cells inhibited proliferation and promoted apoptosis.. Additionally, we identified SMAD5, previously reported to regulate cell apoptosis, as a potential target of miR-27a-3p (37,38). We investigated this by Western, luciferase reporter assay, and flow cytometry. Our

results suggest that miR-27a-3p may target SMAD5 to promote apoptosis in KGN cells, which may suggest a potential role in PCOS pathogenesis due to GC apoptosis.

An earlier report showed that miR-23a and miR-27a promote human granulosa cell apoptosis by targeting SMAD5 (26). However, our results differ from those results in several ways. Nie et al. found that miR-27a-3p is upregulated in patients with premature ovarian failure (POF), but PCOS patients were excluded from their analysis. In our study, we focused on PCOS patients. We found that miR-27a-3p was upregulated and SMAD5 was downregulated in PCOS GCs. In contrast to normal GCs or KGN cells, due to insulin resistance, the expression of miR-27a-3p in PCOS GCs was not affected by insulin. Normal GCs or KGN cells responded to insulin treatment with decreased expression of miR-27a-3p and increased expression of SMAD5, and both these changes decreased apoptosis. Our data also showed that insulin levels in KGN cells downregulated the expression of miR-27a-3p and that this down-regulation occurred *via* STAT1 and STAT3, which was not reported previously.

GCs are the main source of steroidogenesis, including production of E_2 and P_4. Gonadal steroids play key roles in ovarian follicular development, oocyte maturation, and ovulation (39). Some miRNAs were recently reported to influence steroid hormone release by ovarian GCs (40,41). In the present study, we observed that miR-27a-3p overexpression in KGN cells had no significant effect on E_2 and P_4 secretion. Thus, miR-27a-3p may utilize a strategy other than hormone secretion to affect GC function.

It is well known that insulin plays a key role in the regulation of ovarian function, and stimulates the proliferation and hormone production of GCs (22,42). Several miRNAs have been reported to be regulated by insulin in PCOS (29). Jiang et al. reported that high concentrations of insulin induced the upregulation of miR-93, stimulated KGN cell proliferation, and reduced CDKN1A expression in PCOS (28). Xiang et al. observed that insulin treatment in KGN cells inhibited miR-483, and promoted IGF1 and cell proliferation (29). In this work, we found that insulin could downregulate the expression of miR-27a-3p and inhibit apoptosis in KGN cells. The mechanism explaining the changes mediated by insulin in apoptosis and the effects on the TFs upstream of miR-27a-3p, STAT1 and STAT3, require further study. Additionally, further clinical investigations of miRNA expression for a large group of PCOS patients are needed.

Hyperandrogenism is a key endocrine characteristic of PCOS patients (43). Androgens are mainly produced in adrenal glands and ovaries, but the hyperandrogenism of PCOS is closely related to the ovaries (44). Androgens via the androgen receptor (AR) to induce induced the expression of miR-125b in mouse GCs, KGN cells, and GCs from women undergoing in vitro fertilization (45). Similarly,,miR-27a-3p expression is regulated by androgen in prostate cancer, both transcriptionally and post-transcriptionally (46), Another study demonstrated activity of miR-27a-3p as an androgen-induced tumor suppressor, targeting MAP2K4 in prostate cancer (34). Although miR-27a-3p has been proposed to be an androgen-response miRNA, this analysis was not within the scope of this study.

Taken together, the first part of our study demonstrated significantly high levels of miR-27a-3p expression in PCOS GCs and ovary. The last part of the study showed the role of miR-27a-3p in the downregulation of SMAD5 and induction of apoptosis. However, there were contradictions between the findings in the first and last part of this study, as insulin downregulated microRNA-27a-3p expression in KGN cells, but samples from PCOS patients presented with higher levels of insulin and higher levels of miR-27a-3p expression. Thus the question is, if insulin decreased miR-27a-3p expression in GCs, how did the increased insulin level increase miR-27a-3p expression? Insulin-stimulated primary GCs isolated from PCOS patients, in contrast to normal GCs or human ovarian granulosa-like tumor cells (KGN cell line), did not respond to decreased miR-27a-3p expression. This lack of decreased miR-27a-3p expression in GCs of PCOS patients displaying increased insulin levels could be explained by the insulin resistance of GCs. In the regulation of miR-27a-3p expression, KGN cells exhibited characteristics of normal human GCs. Although the KGN cell line is a useful model to understand the regulation of steroidogenesis, cell growth, and apoptosis in human GCs (21), it might not be a good model to study GC functions in the context of insulin resistance or hyperinsulinemia.

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Author contribution:

ST and XL designed the study concept; M.W, J.S, MC, J.G. and M.B performed the experiments; all the authors (MW, JS, BX, MC, JG, YX, MB, HZ, LP, FS, NR, SW, ST, XL) analyzed and interpreted the results; MW, MC, NR and XL drafted the manuscript and all the authors have approved the final manuscript.

Disclosure Statement:

I certify that neither I nor my co-authors have a conflict of interest that is relevant to the subject matter or materials included in this work.

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Figure 1 (black and white) Expression of miR-27a-3p and other miRNAs in human GCs of non-treated and treated PCOS and normal healthy patients. (A-H) The expression of miR-27a-3p, miR-27a-5p, miR-23a, and miR-24-2 in excised GCs of 12 healthy control and 21 PCOS patients undergoing IVF treatment and 13 non-PCOS cuneiform ovarian resections and non-treated 17 PCOS. MiR-27a-3p expression in GCs (A) and ovaries (B) in the PCOS group compared with the control group. MiR-27a-5p (C, D), miR-23a (E, F), miR-24-2 (G, H) expression in the both groups. PCOS OV, PCOS ovarian. *, $P < 0.05$; **, $P < 0.01$

Figure 2 (black and white) Effects of insulin treatment on miR-27a-3p in KGN cells. (A) The expression of miR-27a-3p in KGN cells with different amounts of insulin. KGN cells were stimulated for 24 h without (0) or with 1, 10, and 100 ng/mL of insulin. (B) The predicted binding sites of STAT1 and STAT3 by JASPAR software in the 2000 nucleotides upstream of the miR-27a-3p. (C) The relative expression level of miR-27a-3p in STAT1-overexpressed cells and controls. (D-F) Protein levels (D) and quantification of STAT1 (E) and STAT3 (F) in insulin-treated KGN cells. *, $P < 0.05$; **, $P < 0.01$

Figure 3 (black and white) Effects of miR-27a-3p on cell proliferation and apoptosis in KGN cells. (A) The expression levels of miR-27a-3p in KGN cells after transfection with the miR-27a-3p mimic. (B) Cell viability of KGN cells. (C) Apoptosis rate of KGN cells. (D) Quantification of cell cycle (the rate of G0/G1 and G2/M). (E) The protein levels of PCNA in KGN cells. (F) Expression of cell cycle-related genes (CDK6, cyclin D1, and cyclin E) in KGN cells. (G) Luciferase activity of SMAD5 gene 3'-UTR. (H) The mRNA level of SMAD5 in KGN cells. (I) The protein level of SMAD5 in KGN cells. (J-K) The mRNA expression level of SMAD5 in primary PCOS GCs (J) and OV (K). (L) The mRNA expression level of SMAD5 in

PCOS GCs from public database. GAPDH was used to normalize the expression level. *, $P < 0.05$; **, $P < 0.01$.

Figure 4 (black and white) Effects of knockdown/ overexpression of SMAD5 on KGN cells. (A, B) The mRNA (A) and protein (B) levels of SMAD5 in KGN cells after si-SMAD5 transfection. (C) Cell viability of KGN cells after si-SMAD5 transfection. (D) Apoptosis rate of KGN cells after si-SMAD5 transfection. (E, F) The mRNA (E) and protein (F) levels of SMAD5 in KGN cells after pSMAD5 transfection. (G) Cell viability of KGN cells after pSMAD5 transfection. (H) Apoptosis rate of KGN cells after pSMAD5 transfection. *, $P < 0.05$; **, $P < 0.01$

Figure 5 (black and white) Effects of insulin treatment on apoptosis and SMAD5 expression in KGN cells. (A) Cell viability of KGN cells treated with 100 ng/mL of insulin for 48 h. (B) Quantification of the apoptotic effect in KGN cells treated with insulin for 48 h. (C) Effects of 100 ng/mL insulin 48 h treatment on SMAD5 protein expression in KGN cells. (D) The relative expression levels of SMAD5 protein were normalized to GAPDH. *, $P < 0.05$; **, $P < 0.01$

Figure 6 (black and white) Expression of miR-27a-3p in primary GCs treated with insulin. The expression level of miR-27a-3p in GCs from PCOS and normal healthy patients treated with different doses of insulin. *, $P < 0.05$; **, $P < 0.01$

Table 1 **Characteristics of the subjects in the study**

Table 1 **Characteristics of the subjects in the paper.**

	Control group (n=13)	PCOS group (n=21)	P value
Age (years)	30.00 ± 0.74 [2.77]	28.67 ± 0.675 [3.698]	0.24
BMI (kg/m^2)	23.63+ 1.701	24.69+ 1.193	0.5670
Testosterone [nmol/L]	1.30 ± 0.15 [0.57]	1.95 ± 0.13 [0.63]	0.0036
FAI	2.16 ± 0.39 [1.46]	3.88 ± 0.61 [2.77]	0.04
Glucose [mmol/L]	4.52 ± 0.2 [0.75]	4.59 ± 0.16 [0.63]	0.77
Insulin [μU/mL]	4.22 ± 0.28 [1.03]	8.53 ± 1.12 [4.86]	0.0028
HOMA	0.84 ± 0.06 [0.22]	1.77 ± 0.25 [1.08]	0.0034
SHBG [nmol/L]	83.39 ± 12.79 [47.85]	75.64 ± 10.51 [49.31]	0.64
DHEA-S [μmol/L]	6.43 ± 0.52 [1.94]	7.92 ± 0.52 [2.46]	0.06
Hyperandrogenism , (n)%	4 (28,6%)	15 (65,2%)	
Normal androgen, (n)%	10 (71,4%)	8 (34.8%)	

Abbreviation: BMI, Body Mass Index; FAI, Free Androgen Index; SHBG, Sex Hormone-Binding Globulin; DHEA-S, Dehydroepiandrosterone Sulfate; HOMA, Homeostasis Model Assessment.

Data are presented as Mean ± SEM [SD]

Peptide/protein target	Antigen sequence (if known)	Name of Antibody	Manufacturer, catalog #, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used	RRID (required in revised MSs)
SMAD5		SMAD5 Antibody	CST #9517S	Rabbit; polyclonal	1/1000	AB_10699149
PCNA		PCNA (PC10)	Santa Cruz Biotechnology sc-56	Mouse monoclonal	1/1000	AB_628110
GAPDH		GAPDH (14C10)	CST #2118	Rabbit; monoclonal	1/1000	AB_561053
STAT1		P84/P91 (E-23)	Santa Cruz Biotechnology sc-346	Rabbit; polyclonal	1/1000	AB_632435
STAT3		124H6	CST #9139s	Mouse; monoclonal	1/1000	AB_10694804