miR-132 is up-regulated in polycystic ovarian syndrome and inhibits granulosa cells proliferation via targeting Foxa1

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Research

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Abstract

Background: Polycystic ovary syndrome (PCOS) is one of the most common endocrine metabolic disorders characterized by hyperandrogenism, polycystic ovaries and ovulatory dysfunction. Several studies have suggested that the aberrant expression of miRNAs serves an important role in the pathogenesis of PCOS, though the role and underling mechanism of microRNA-132 (miR-132) in the development of PCOS remain unclear.

Methods: The expression of miR-132 in granulosa cells (GCs) derived from 26 PCOS patients and 30 healthy controls was detected through RT-qPCR. And the apoptosis levels of granulosa cells were measured by TUNEL.Granulosa-like tumor cell line (KGN) was cultured for cell counting kit-8 (CCK-8) was assays after over-expression of miR-132 or knockdown TargetScan was applied to analysis the potential targets of miR-132, which was further verified by luciferase assay, RT-qPCR and western blot.

Results: The expression of miR-132 was declined in granulosa cells of PCOS patients. Meanwhile, the significantly increased apoptotic nuclei were present GCs of PCOS patients. Furthermore, over-expressed of miR-132 inhibited the proliferation of KCN cells. In addition, our results verified that miR-132 directly targeted Foxa1, knockdown of which suppressed KGN cells proliferation.

Conclusion: Our results revealed that miR-132 inhibits the cell viability and induces apoptosis by directly interacting with Foxa1, indicating a role of miR-132 to be a potential target in the PCOS patients.

Introduction

Polycystic ovary syndrome (PCOS) is one of the most prevalent endocrine metabolic disorders affecting about 5-10% women of reproductive age [1, 2]. It is generally characterized by hyperandrogenism, polycystic ovary and anovulation, but also associated with metabolic dysfunction, cardiovascular risk, abnormal granulosa cells (GCs) proliferation as well as arrest of follicle growth [3, 4]. The clinical symptoms and signs of PCOS vanished or irregular menstruation, difficulty in pregnancy, acne, as well as thick, dark and smooth skin patches [5]. Though, genetic and environmental factors play a major role in the pathogenesis of PCOS, however the underlying molecular mechanism is still elucidated [6].

MicroRNAs (miRNAs) are are small RNAs without the protein coding function, which function in negatively regulating protein-coding gene expression through RNA silencing and post-transcriptional regulation [7, 8]. Studies have shown that aberrant expressions of miRNAs are associated with the pathological progression of various diseases, including cancer, metabolic diseases, inflammation and reproductive disorders [8]. However, the function and underlying molecular mechanism of miRNA in follicular development and the development of PCOS has not yet been entirely clear.

miR-132, located in the intron of a non-coding gene on chromosome 17 in humans, has been reported to play different roles in various diseases [9-11]. Recently, studies have demonstrated that miR-132 can mediate tumor initiation and development through regulating cancer cell proliferation, apoptosis, invasion

and migration [12, 13]. Furthermore, Shaogen Wu et al., revealed that miR-132 could promote estradiol synthesis in ovarian granulosa cells via translational repression of Nurr1 [14]. However, the role and mechanism of miR-132 in PCOS are still unclear. Therefore, the aim of present study is to explore whether miR-132 are involved in the abnormal proliferation of PCOS GCs and its potential mechanism.

Materials And Methods

Patients and samples

The study population consisted of women referred to Reproductive Medicine Center of Shanxi Women and Infants Hospital, Taiyuan, China between June 2016 and December 2016. All subjects were Han ethnic, from the Shanxi Province, in north China. The study was approved by the Ethics Committee of Shanxi Women and Infants Hospital, and all the participants signed a written informed consent for participation in this study. The blood and follicular fluid samples were obtained from 26 PCOS patients and 30 healthy controls. Diagnosis of PCOS was based on Rotterdam Criteria [15], including oligo-ovaluation and/ or anovulation, excess androgen activity, and ultrasound image of polycystic ovaries. Patients with endometriosis, congenital adrenal hyperplasia, hypothyroidism, androgen-secreting tumors, Cushing's syndrome and other systemic diseases were excluded from the study. The controls were infertility patients due to tubal and/or male factors with regular menstrual cycle. All participants accepted endocrine tests and other routine checks and the results are listed in **Table1**.

Controlled ovarian hyperstimulation protocol

Both PCOS patients and control patients received in vitro fertilization and embryo transfer (IVF-ET) treatments, following standard operation procedure (SOP). All women underwent controlled ovarian hyper-stimulation (COH) with gonadotropin releasing hormone (GnRH) agonist long protocol commenced pituitary suppression with leuprolide acetate (Dipherelin 0.1mg, GenSci Company, Chang Chun, China) at a dose of 0.05mg/d, during the mi-luteal phase of the preceding cycle. Complete pituitary suppression was comfired by a serum FSH level<5mIU/ml, LH level<5mIU/ml, E2 level<50pg/ml, bilateral antral follicle diameter <5mm, endometrial thickness ≤5mm. Urofollitropin (LiZHu, China) were used at doses ranging between 75IU/day and 300IU/day in accordance with patient age, body mass index, size and number of antral follicles, and serum basic FSH level. The dosage of urofollitropin was adjusted according to ovarian reponse, which was assessed by ultrasound and serum E2 levels. Recombinant human horogonadotropin-afa solution (hCG, Merck Serono S.p.A, Italy) was administered subcutaneously at the 250μg dosing level when at least two follicles with ≥18mm average diameter were detected. Oocyte retrieval was performed under the guidance of transvaginal ultrasounds 34 to 36 hours after the hCG injection. Human granulosa cells (GCs) were obtained from follicular fluid at the same time.

Cell culture

After oocyte retrieval, all follicular fluids from each patient were pooled and stored in a tube. The GCs were parade and cultured as described previously [16]. Briefly, the aspirated follicular fluid was

centrifuged at 1000g for 10 minmutes after removal of oocytes. The cell pellet was resuspended in 1ml phosphate buffer saline (PBS). Then, the suspension was overlayed on 1ml Ficoll, and centrifuged at 800g for 30 min. GCs were aspirated from the interface and washed a few times with PBS. Next, the isolated and purified GCs were cultured in Dulbecco's modified Eagle medium/nutrient mixture F12 Ham medium (DMEM/F12, Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, TBD, Tianjing, China), at 37℃ with 5% CO_2 and 95% humidity.

Cell transfection

Human granulosa-like tumor cell line, KGN cells were from the American Type Culture Collection (ATCC, VA, USA), which maintained the physiological characteristics of ovarian cells. The cells were grown in (DMEM/F12, Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, TBD, Tianjing, China), at 37℃ with 5% CO_2 and 95% humidity. The miRNA-132 mimics, inhibitor, and negative control (NC) were designed and synthesized by Qiagen (Valencia, California). Foxa1-siRNA, Foxa1-pcDNA3.1, pcDNA3.1 empty vector and negative control (si-NC) were designed and synthesized from Gene-Pharma (Shanghai, China). The cells were seeded in 6-wel plates (2×10^5 cells/well) 1day before transfection to reach the confluency of 90%, and then the medium was replaced by serum- and antibiotic-free medium. Then, miR-132 mimic, miR-132 inhibitor, and siFoxa1 were transfected at a final concentration of 50 nM using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, California) following the manufacturer's protocols. At 36 hours after transfection, the cells were collected for the following assays.

Cell proliferation assay. Cell proliferation was assessed using the CCK-8 method. For the effect of miRNA-132 or Foxa1 on proliferation, cells transfected with miRNAs or siRNAs were plated in 96-well plates at 5×10^3 cells/well. Cell proliferation was detected at 24, 48, and 72h after transfection using CCK-8 at 45nm according to the protocol of the manufacturer's instructions (Beyotime Institute of Biotechnology, Shanghai, China) at 37℃ for 4h.

Tunel Assay. Granulosa cell were cultured directly on coverslips. After the treatment, the granulosa cells' apoptosis was determined by TUNEL staining using an TUNEL cell apoptosis detection kit (KeyGEN BioTECH, Nanjing, China), according to the manufacturer's recommendations. Images were taken using an Olympus microscope (BX40, Tokyo, Japan). Five photos (magnification ×200) were taken randomly for each sample. Data are reported as the percentage of TUNEL-positive cells among the total number of cells as described. Each experiment was performed in triplicate.

RNA isolation and qRT-PCR assay. Total RNA of GCs was extracted using the RNeasy/miRNeasy Mini kit (Qiagen, Limburg, The Netherlands) according to the manufacturer's instructions. Total RNA (2ng) was used for reverse transcription, using the one-step RT-PCT kit (Qiagen, Limburg, The Netherlands) following the manufacturer's instructions. The primers for miR-132 were the exact sequence of mature miR-132. U6 was used as the internal control. They were purchased from Qiagen (Limburg, The Netherlands). The conditions for miRNAs as follows (40 cycles): 95℃ for 15min, followed 95℃ for 15seconds, and 60℃ for 1min. The primer sequences for Foxa1 and GAPDH were as follows: Foxa1 (sense, 5'-

AGGGCTGGATGGTTGTAT TG-3’; antisense, 5’-GCCTGAGTTCATGTTGCTGA-3’); GAPDH (sense, 5’-GAAGGTGAAG GTCGGAGTC-3’; antisense, 5’-GAAGATGGTGATGGGATTTC-3’) was used as an internal control. All reactions were run in triplicate and gene expression was determined by $2^{-\triangle\triangle Ct}$.

Dual-Luciferase Reporter Assay. The Foxa1-3’ UTR and the Foxa1-mutated-3’UTR fragment were amplified by PCR. Then, the fragment was inserted into the pGL3 luciferase promoter vector (Promega, Madison, WI, USA) to develop the Luc-pGL3-Foxa1-3’UTR and Luc-pGL3-Foxa1-mut-3’UTR vectors. Cells in 24-well plates were contransfected with Luc-pGL3-Foxa1-3’UTR or Luc-pGL3-Foxa1-mut-3’UTR vector and miR-132 mimics or miR-NC using Lipofectamine 2000 regent (Invitrogen) according to the instructions of the manufacturer. The Renilla luciferase reporter vector was transfected as an internal control in each assay. At 48h post-transfection, firefly and Renilla luciferase activities were detected using a dual-luciferase reporter system (Promega, Madison, WI, USA). The results are expressed as relative luciferase activity (Firefly Luc/Renilla Luc). All experiments were performed 3 times in triplicate.

Western blot analysis. Western blot analysis was applied to evaluate levels of Foxa1. Briefly, the cells were collected and lysed on ice in RIPA lysis buffer (Beyotime, Beijing, China) with protease inhibitor according to the manufacturer’s instructions. The protein concentration of cell lysates was determined using BCA kit (Boster, China). Equal amounts of protein lysates (30μg each lane) was resolved by 10% SDS-PAGE, and then electrotransferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked with TBST containing 5% non-fat milk for 2h at room temperature, and then incubated with the specific antibodies at 4℃ overnight, including mouse anti-Foxa1 (1:1000; ab40868) monoclonal antibodies and mouse anti-GAPDH (1:200; ab8245) monoclonal antibodies (All from Abcam, Cambridge, MA, USA). After washing with TBST, The membranes were further incubated with HRP-labelled goat anti-mouse IgG (1:2000; cat. no. BA1051; Boster biological engineering co., LTD; Wuhan; China) at 37℃ for 1h, followed by chemiluminescence for visualization with an ECL kit (KeyGEN BioTECH, Nanjing, China).

Statistical analysis. All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad software, Inc., USA). For the analysis of endocrine tests, results were indicated as mean±standard error of the mean (SEM). Student’s t-test was performed for comparisons of the mean values of two groups; one-way ANOVA was used to determine differences among the mean values of more than two groups because the quantitative data followed a normal distribution.. Differences between groups were considered statistically significant at a p-value of < 0.05.

Results

Patient characteristics. The characteristics of females in PCOS group and control group are summarized in **table 1**. In total, 26 cases of PCOS patients were recruited from all patients seeking reproductive assistance at the Reproductive Medicine Center of Shanxi Women and Infants Hospital. Each PCOS patient was confirmed clinically. 16 cases of tubal infertility and 14 cases of male factors infertility were included as controls for this study. There was no significant difference between the PCOS and control

groups regarding age, infertility duration, FSH, E_2 and PRL. However, compared with control group, the BMI, LH, TES were significantly increased (P<0.05)

miR-132 expression is down-regulated in human GCs from PCOS. RT-qPCR was performed to detect miR-132 expression in Granulosa cells of 26 PCOS patients and 30 controls. Compared with the controls, the expression of miR-132 was significantly up-regulated in the GCs of patients with PCOS (P<0.05; **Figure 1**).

Granulosa cells from PCOS patients have lower apoptotic rates. In order to determine the granulosa cell apoptotic rate in PCOS, cells were cultured directly on coverslips and stain with TUNEL cell apoptosis detection kit. Significantly increased apoptotic nuclei were present in PCOS group as compared to the control group (P<0.05; **Figure 2**)

miR-132 negatively regulates cell growth and proliferation in granulosa cells. Having noted a significantly higher expression of miR-132 in granulosa cells of the ovaries from PCOS patients, we were interested to test the hypothesis that miR-132 might be associated with the growth and proliferation in granulosa cells. As shown in **Figure 3A**, the CCK-8 assay revealed that miR-132 decreased expression in GCs led to a promotion in proliferation **(**P<0.05). In contrast to the miR-132 mimics, the miR-132 inhibitor promoted cell growth (**Figure 3A**; P<0.05). To further determine miR-132 on cell apoptosis in human GCs, western blotting was used to detect the protein expression levels of Bax and Bcl-2. The results showed that mirR-132 mimics significantly increased Bax protein levels and decreased the protein level of Bcl-2 when compared with the control group. However, miR-132 inhibitor remarkedly upregulated the protein level of Bcl-2 and diminished Bax protein level when compared with the control group (**Figure 3B and C**; P<0.05).

The miR-132 directly inhibited Foxa1 expression by binding to its 3'-UTR. Foxa1 was predicted to be a target of miR-132 by the online database TargetScanHuman 7.1 (www.targetscan.org), with the sequence GACUGUUA in its 3'UTR being the predicted binding site (**Figure 4A**). Remarkable downregulation of Foxa1 was detected by qRT-PCR in human granulosa cells from PCOS (**Figure 4F;** P<0.05). Then, we performed qRT-PCR and western blotting to observe the expression of Foxa1 on mRNA and protein levels in granulosa cells transfected with miR-132 mimics and inhibitor. Compared to controls, the mRNA and protein levels of Foxa1 were significantly decreased after upregulation of miR-132, however, were remarkably increased after downregulation of miR-132 (**Figure 4C, D and F;** P<0.05). To further demonstrate whether Foxa1 was a direct target of miR-132, Foxa1 3'-UTR was cloned into a luciferase reporter vector and the putative miR-132 binding site in the Foxa1 3'-UTR was mutated **(Figure 4E**). Taken together, these data suggest that Foxa1 gene is a direct target of miR-132 overexpression, which inhibits Foxa1 expression in GCs.

Silencing of Foxa1 inhibits Human GCs proliferation. To explore the function of Foxa1 in granulosa cells, we knocked down Foxa1 by siRNA in human granulosa cells from control patients. qRT-PCR analysis and western blotting analysis indicted that mRNA and protein levels of Foxa1 was significantly decreased after 24 hours in GCs transfected with Foxa1-siRNA (**Figure 5A, B and C;** P<0.05). Then, CCK-8 assay was performed to evaluate the effect of Foxa1-siRNA on human GCs. As expected, Foxa1 siRNA-transfected

GCs displayed a reduced cell survival rate compared to si-NC at 48h posttransfection (**Figure 5D;** $P<0.05$). These results indicated that silencing Foxa1 inhibits human GCs growth.

Foxa1 overexpression abrogates the suppressive effect of miR-132 mimics. To further determine the role of miR-132 on cell proliferation through the direct targeting of Foxa1 in human GCs, cells were transfected with pcDNA3.1-Foxa1or miR-132 mimics. As shown in **Figure 6A**, overexpression of Foxa1 by pcDNA3.1-Foxa1 significantly increased Foxa1 expression in human GCs ($P<0.05$). At the same time, the suppressive effect of miR-132 mimics on Foxa1 expression was dramatically reversed by Foxa1 overexpression. Subsequently, we found that the suppressed cell proliferation by miR-132 mimics transfection was attenuated by Foxa1 overexpression in human GCs cells (**Figure 6B;** $P<0.05$).

Discussion

The aim of this study was to explore whether miR-132 was involved in the abnormal proliferation of PCOS GCs and its underlying mechanism. In our study, we observed that the expression of miR-132 was significantly decreased in GCs from patients with PCOS. Furthermore, our results showed that decreased miR-132 was associated with decreased cell apoptosis index of GCs from PCOS. Moreover, dual-luciferase reporter assay showed that Foxa1 is a direct target for miR-132 and promotes GCs proliferation. The Foxa1 overexpression can abrogate the suppressive effect of miR-132 mimics. These results indicate that miR-132 can suppress cell proliferation, and the potential underlying mechanism is associated with targeting and suppressing Foxa1 expression.

Recent studies have shown that miR-132 plays a proapoptotic role in many cancer types, such as glioma [17], colorectal cancer [18] [19], osteosarcoma [20], hepatic carcinoma [21], breast cancer [22], pituitary tumor [23], lung cancer [24]. In addition, miR-132 has been shown to be up-regulated in periovulatory mouse GCs after LH/hCG treatment and to enhance estradiol synthesis in GCs [14, 25]. However, a large number of studies about miR-132 were mainly focused on cancer, the mechanism of miR-132 function of PCOS is not investigated. This was first time to confirm that miR-132 were involved in inhibiting the proliferation of KGN cells, suggesting that miR-132 play crucial roles in the abnormal proliferation of GCs, which might lead to PCOS.

Forkhead box protein A1 (FOXA1) is a transcription factor that belongs to the forkhead family consisting of the winged-helix DNA-binding domain and the N-terminal and C-terminal transcriptional domains, thereby delineating genomic regions and allowing for subsequent binding of other transcription factors, such as the ER, progesterone receptor (PR), and androgen receptor (AR) [26-28]. FOXA1 is expressed in various organs, including breast, liver, pancreas, and prostate, and can influence the expression of a large number of genes associated with metabolic processes, regulation of signaling, and the cell cycle [29, 30]. It has been reported that Foxa1 was a direct target of miR-132 in breast cancer, thyroid cancer, and nasopharyngeal carcinoma [31-33]. In consistent with the above results, our research further proved that FOXA1 was a direct target of miR-132 in KGN cells proliferation. However, Qing Sang et al., who identified microRNAs in human follicular fluid of PCOS, showed that miR-132 are expressed at significantly lower

levels in the follicular fluid of polycystic ovary patients than in healthy controls [34]. The differences were likely due to different sources. The miR-132 studied above was isolated from human follicular fluid, while our miR-132 isolated from human GCs. In addition, as far as we know, the regulation process of GCs proliferation was complex and closely regulated to various factors [35, 36].

In conclusion, these data demonstrated that the expression of miR-132 was significantly increased in PCOS patients. In addition, over-expression of miR-132 inhibited the proliferation of KGN cells by targeting FOXA1. These results provide new evidence for GCs dysregulation proliferation ovserved in PCOS. Due to the limitation on the number of PCOS samples and cell types, further investigation will be necessary for exploring the underlying molecular mechanisms of miR-132 in POCS.

Abbreviations

CCK-8: Cell counting kit-8; FSH: Follicle stimulating hormone; GCs: Granulosa cells; IVF: In vitro fertilization; LH: Luteotropic hormone; miR-132: microRNA-132; miRNAs: microRNAs; MUT: Mutant type; SOP: Standard operation procedure; COH: Controlled ovarian hyper-stimulation; PBS: phosphate buffer saline; PCOS: Polycystic ovary syndrome; siRNAs: Specific small-interfering RNAs; T: Testosterone; UTR: Untranslated region; WT: Wild type; GnRH: Gonadotropin releasing hormone (GnRH)

Declarations

Acknowledgements

Not applicable.

Author's contributions

Xiangrong Cui and Xuan Jing contributed to the study design and critical revision of the manuscript. Meiqin Yan was in drafting of the manuscript. Junfen Liu and Xueqing Wu contributed to the study and revision of the manscript. Xingyu Bi, Jianrong Cui and Yuan Yuan were involved in the experiment guidance. All authors read and approved the final manuscript.

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Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Ethics approval and consent to participate

This research was approved by the Institutional

Consent for publication

Formal written consent was obtained from each patient.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Characteristics of PCOS patients and the controls

Characteristic	PCOS group (n=26)	Control group (n=30)	*P* value
Age	29.86±2.81	30.11±2.95	0.75
BMI	23.74±3.36	21.84±3.02	0.03
Infertility duration	4.42±2.76	4.78±2.99	0.64
FSH (mIU/ml)	6.79±2.18	6.96±1.92	0.76
LH (mIU/ml)	9.53±6.48	4.37±2.11	<0.001
E_2 (pg/ml)	51.12±14.77	47.96±19.98	0.51
PRL (ng/ml)	14.13±6.48	14.41±6.26	0.87
TES (ng/dl)	1.14±0.39	0.44±0.17	<0.001

All data are expressed as mean±SD. BMI body mass index, FSH follicle-stimulating hormone, LH luteinizing hormone, E_2 estradiol, PRL progesterone, T testosterone, PRL prolactin

Figures

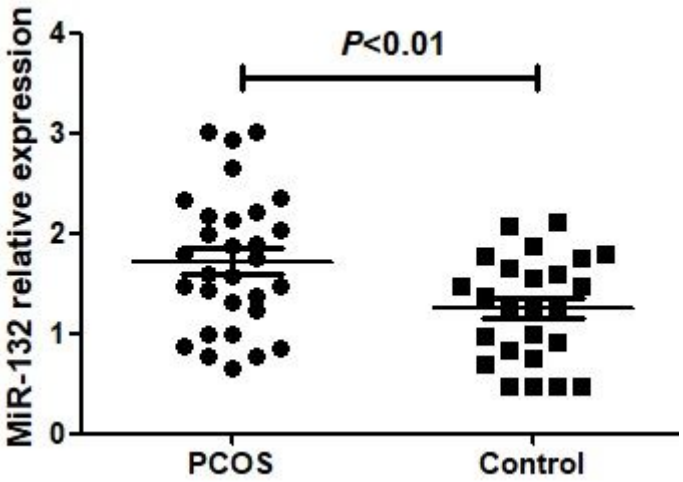

Figure 1

The miR-132 expression is up-regulated in human granulosa cells (GCs) from polycystic ovary syndrome (PCOS). Expression levels of miR-132 were examined by quantitative real-time polymerase chain reaction (qRT-PCR) is isolated human GCs from the aspirated follicular fluid in PCOS (26 cases) and controls (30 cases). *P<0.05 versus controls.

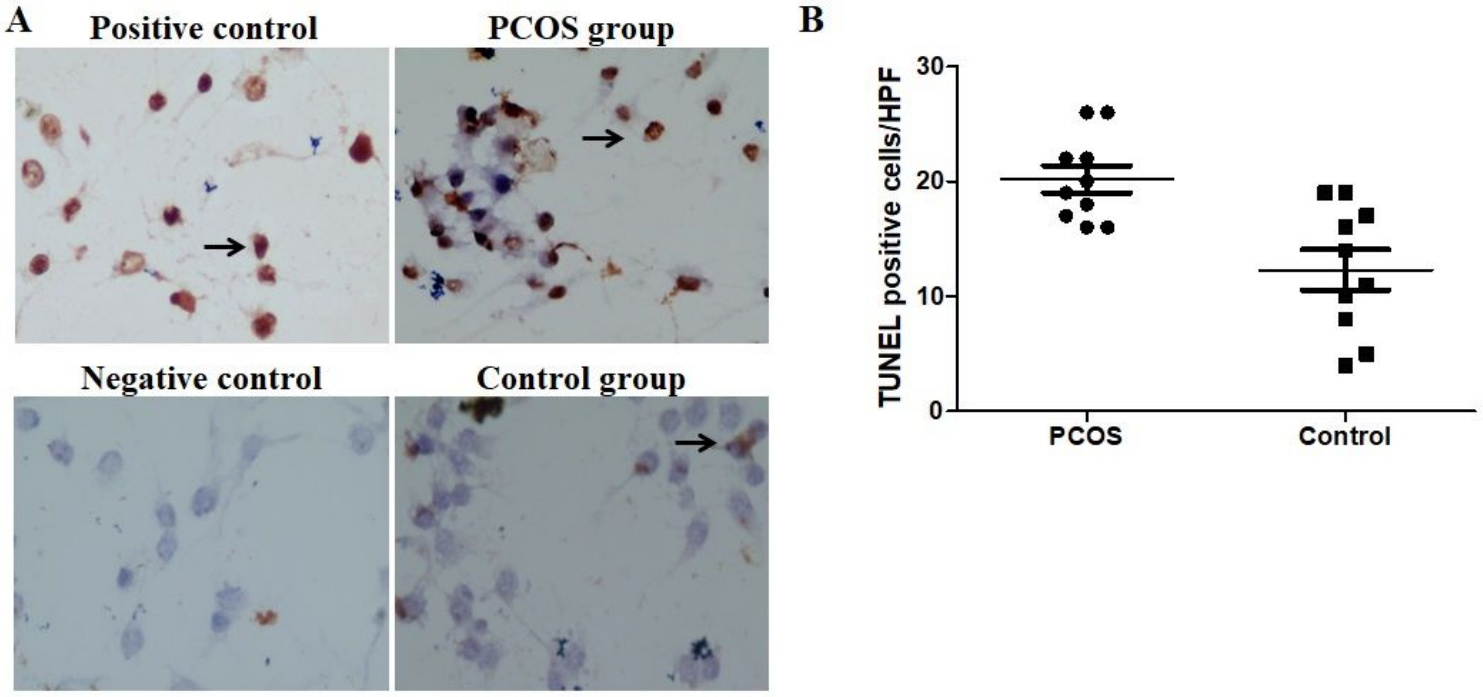

Figure 2

TUNEL assay showing the apoptosis in granulosa cells. The buffy nucelus indicate TUNEL-positive cells. Slides were observed under a microscope (×200).

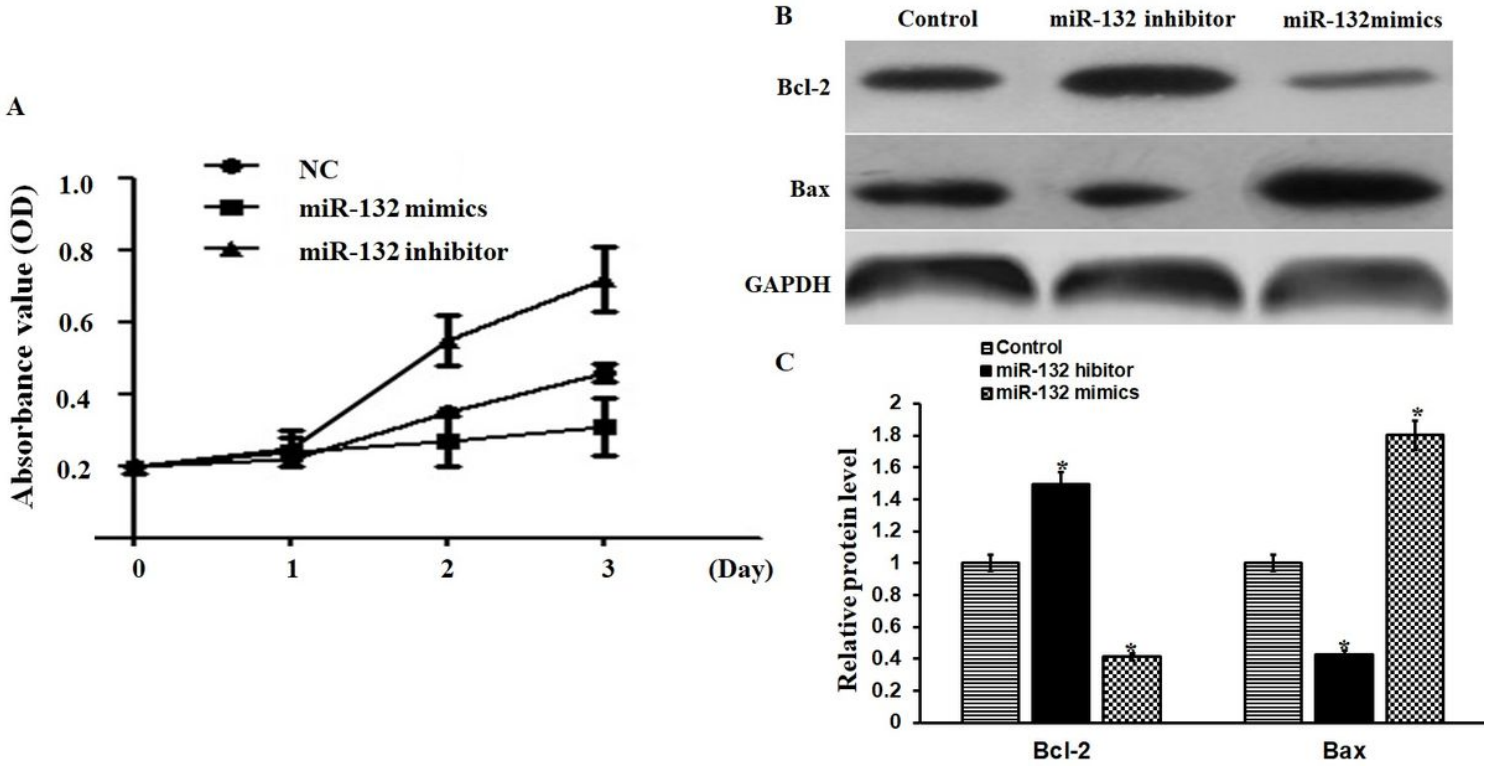

Figure 3

miR-132 negatively regulates cell growth and proliferation in granulosa cells. A. Cell survival rate was determined by CCK-8 assay in isolated human granulosa cells transfected with miR-control, miR-132

inhibitor, or miR-132 mimics. B. Protein levels of Bax and Bcl-2 in granulosa cells were examined by Western blotting with GAPDH as an interal reference. C. A quantitative presentation of the data is shown of protein levels. *P<0.05 versus control.

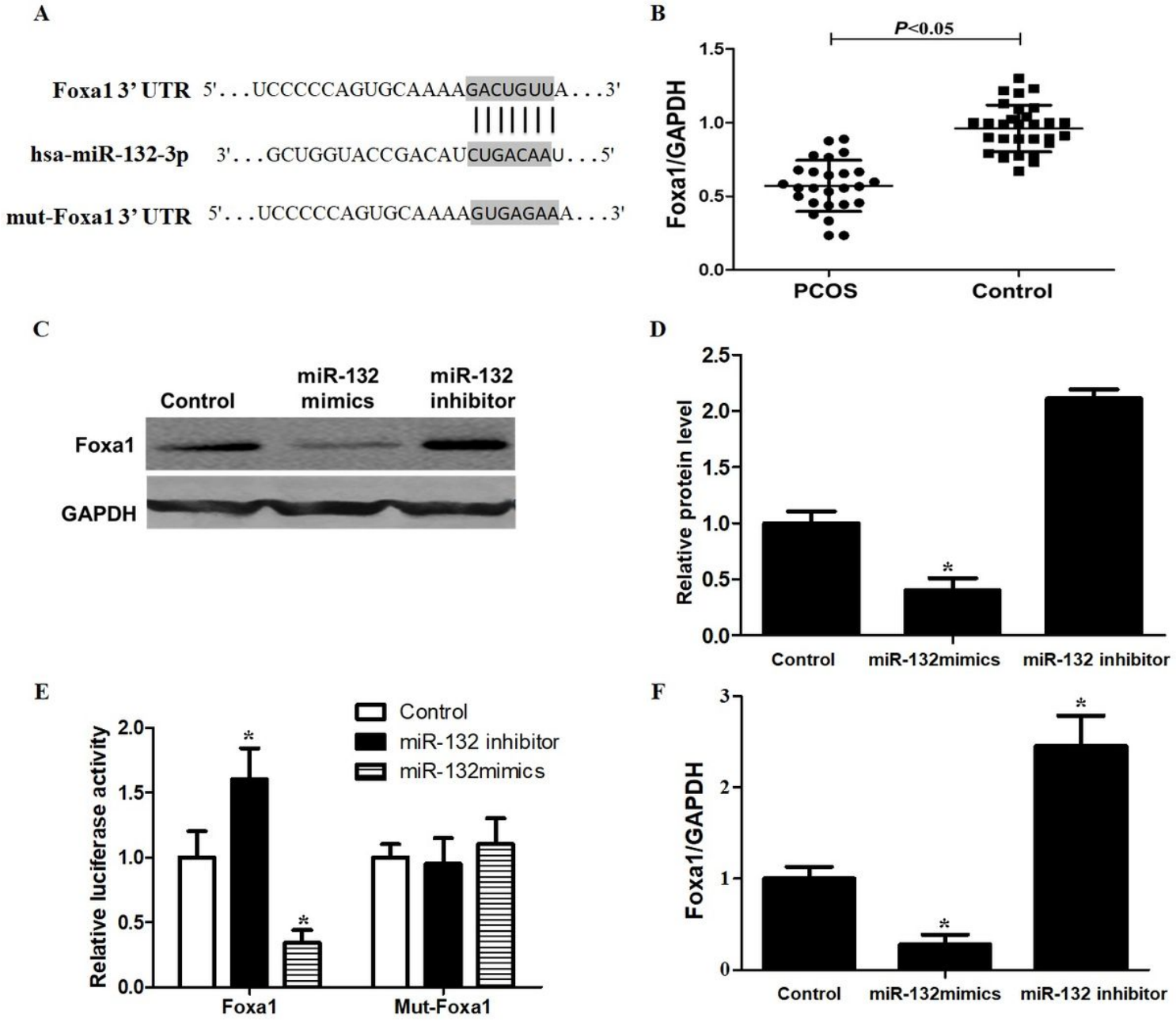

Figure 4

The miR-132 directly inhibited Foxa1 expression by binding to its 3'-UTR. A. The sequence GACUGUUA in Foxa1 3'-UTR is predicted to be the binding site of miR-132. Five bases in the binding site are mutated to construct mut-Foxa1 3'-UTR. B. The mRNA expression level of Foxa1was examined by qRT-PCR in isolated human granulosa cells from the aspirated follicular fluid in PCOS and control patients. C. Western blotting data showed that the protein expression levels of Foxa1. D. A quantitative presentation of the data is shown of protein levels. E. Validation of miR-132 binding to the Foxa1 3'-UTR using a dual-luciferase reporter assay. F. Foxa1 mRNA expression was inhibited by miR-132 overexpression and

upregulated by miR-132 inhibitor. GAPDH is used as an internal reference. GAPDH is used as an internal reference. *P<0.05 versus control.

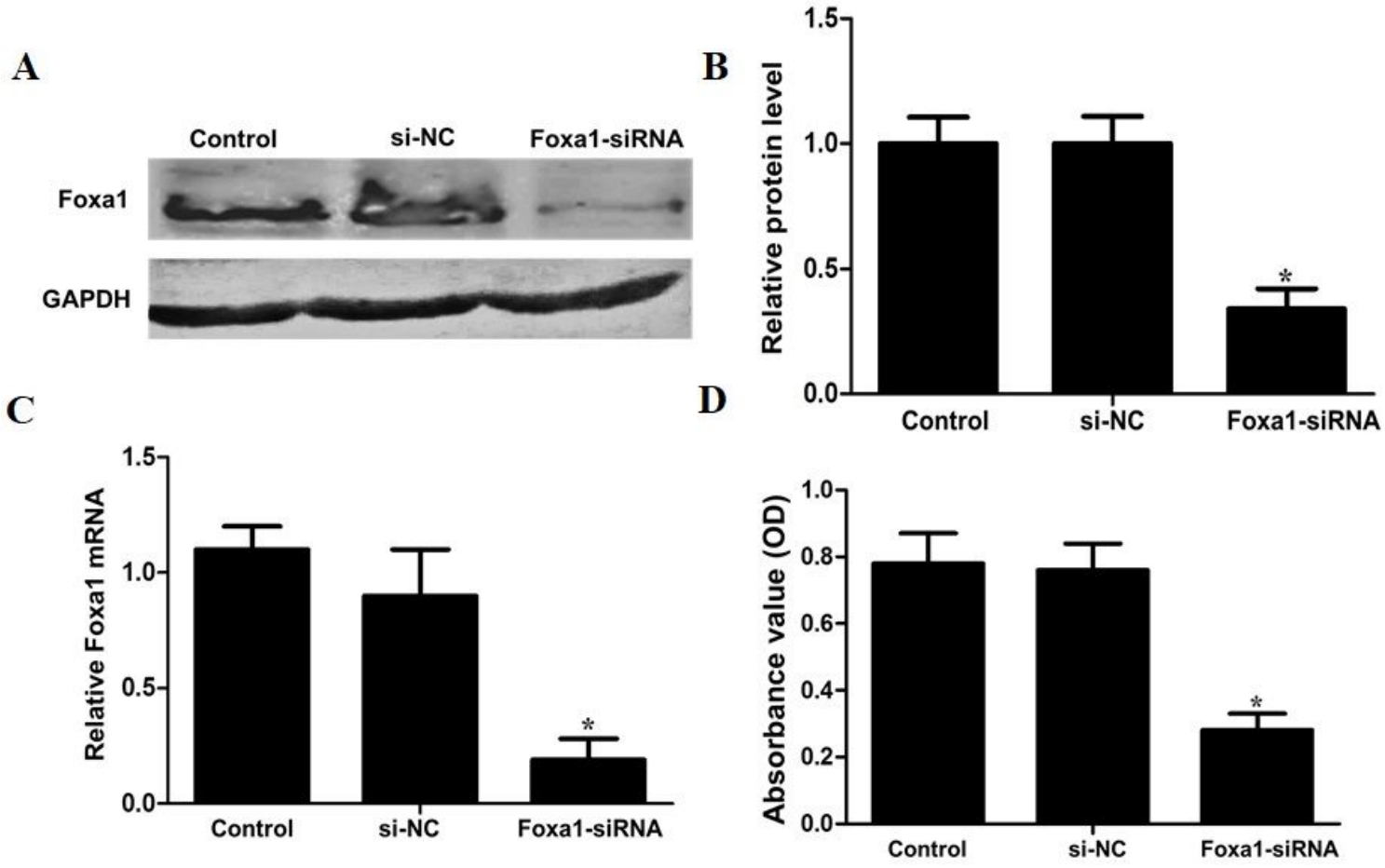

Figure 5

Silencing of Foxa1 inhibits Human GCs proliferation. A. Western blotting data showed that the protein expression levels of Foxa1 in GCs transfected with small interfering RNA (siRNA) Negative control or Foxa1 siRNA. B. A quantitative presentation of the data is shown of protein levels. C. qRT-PCR analysis data showed that mRNA levels for Foxa1 in GCs transfected with small interfering RNA (siRNA) Negative control or Foxa1 siRNA. D. CCK-8 assay was performed to evaluate the cell survival rate of Foxa1-siRNA on GCs. *P<0.05 versus si-NC.

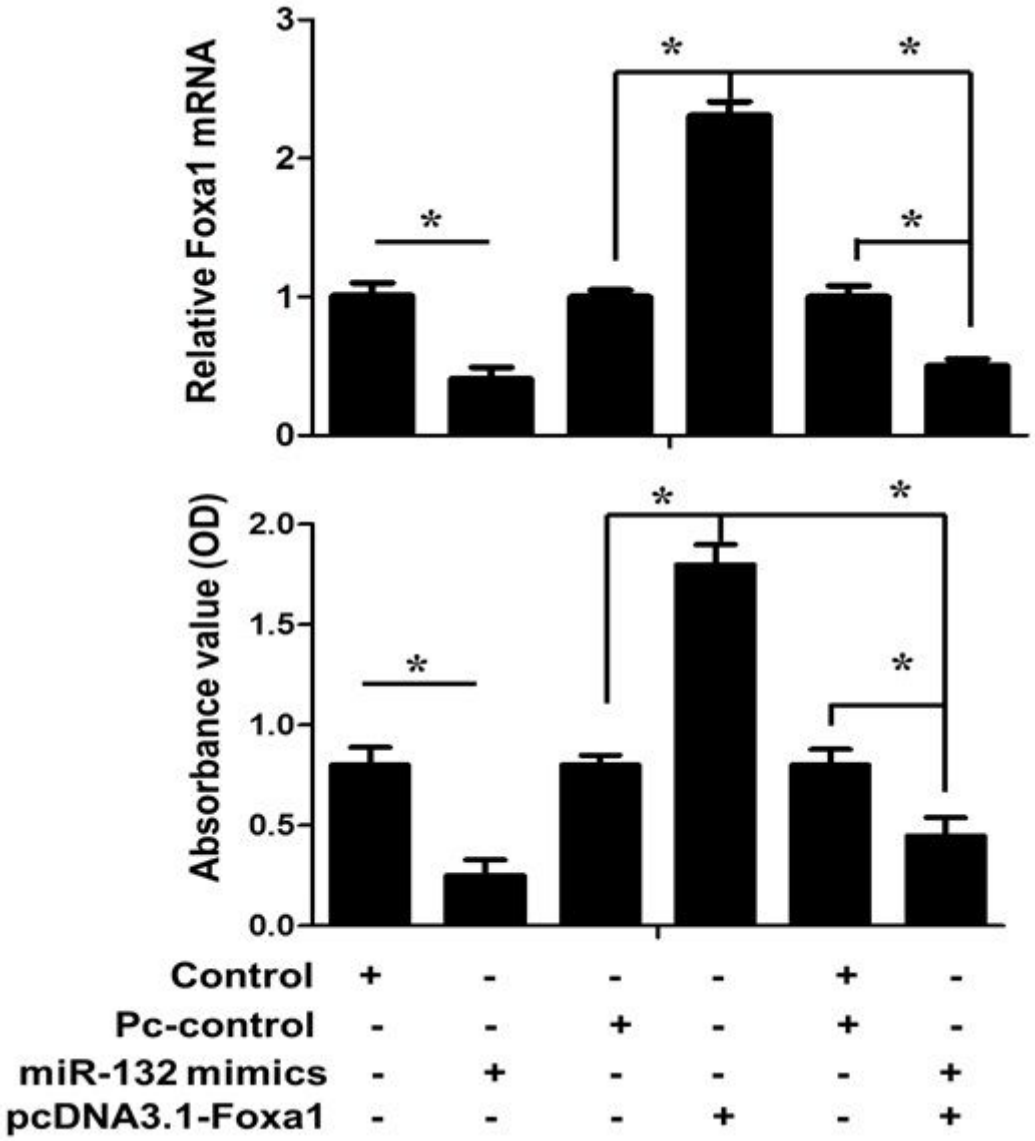

Figure 6

Foxa1 overexpression abrogates the suppressive effect of miR-132 mimics. A. The Foxa1 mRNA expression were tested by qRT-PCR in human granulosa cells (GCs) transfected with miR-132 or pcDNA3.1-Foxa1. B. Cell survival rate was tested by CCK-8 assay. *P<0.05.