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Regular paper

The expression of microRNA-197-3p regulates the proliferation of ovarian granulosa cells through CUL3 in polycystic ovarian syndrome

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MicroRNAs (miR) have been shown to exhibit marked dysregulation in polycystic ovarian syndrome (PCOS). Additionally, granulosa cell proliferation and apoptosis are important in PCOS pathogenesis. Considering this, the current study aimed at characterization of the regulatory function of miR-197-3p on the growth and proliferation of ovarian granulosa cells at the molecular level. The results showed that miR-197-3p is significantly repressed ($P<0.05$) in PCOS tissues and granulosa cells. Granulosa cell proliferation was significantly ($P<0.05$) inhibited by miR-197-3p overexpression *via* induction of apoptosis. *In silico* analysis predicted cullin 3 (CUL3) as the regulatory target of miR-197-3p, which was also confirmed by the dual luciferase reporter assay. CUL3 was overexpressed in KGN ovarian granulosa cells, and its silencing mimicked the tumor suppressive effects of miR-197-3p. Taken together, miR-197-3p exhibits a negative regulatory role in regulating granulosa ovarian cell proliferation *via* posttranscriptional repression of CUL3 in PCOS.

Keywords: polycystic ovarian syndrome, atresia, microRNA, miR-197-3p, apoptosis, Cullin 3

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Abbreviations: CUL3, Cullin 3; CCK-8, Cell counting kit-8; miRs, MicroRNAs; PCOS, Polycystic ovarian syndrome; 3′-UTR, 3′-untranslated regions; WT, Wild-type

INTRODUCTION

Polycystic ovarian syndrome (PCOS) is considered the most common and heterogenous disorder of the female endocrine system with a global incidence of 10–20% among women of reproductive age (Ibáñez *et al*., 2017; Chaudhari *et al*., 2018). Commonly associated ailments with PCOS include menstrual disorder, amenorrhea, hirsutism, chronic anovulation, or even infertility (Wang & Mol, 2017; Martin *et al*., 2017). Obese women with PCOS always show a tendency to develop a variety of metabolic defects, such as lower insulin sensitivity, diabetes mellitus, cardiovascular disease, and fatty liver (Cree-Green *et al*., 2017). Furthermore, women with PCOS reveal comparatively higher risk of experiencing depression and anxiety (Dunaif & Fauser *et al*., 2013). PCOS is currently being managed through the employment of insulin sensitizer drug compounds such as metformin, but the latter has often been shown to exhibit many adverse effects that affect quality of life (Lo *et al*., 2017). Therefore, a need is felt to develop better curative measures against this disorder. In this regard, a number of research investigations have reported that the involvement of dysregulated molecular factors in granulosa cells surrounding oocytes is crucial mediator of PCOS (Sagvekar *et al*., 2017). Currently, different approaches are being developed for the management of this deadly disorder (Barani *et al*., 2020; Nikazar *et al*., 2020; Das *et al*., 2020). Exploring such molecular factors will not only help in better understanding this syndrome but would pave the way towards the formulation of more effective treatment measures for its efficient management (Thai *et al*., 2010).

MicroRNAs (miRs) constitute a heterogeneous class of noncoding single-stranded RNAs that range in size from 20 to 25 nucleotides (Bartel, 2004). The group of RNAs has been deduced to play a variety of molecular functions and regulate crucial cellular aspects such as growth, proliferation, differentiation, and apoptosis (Flynt & Lai, 2008). The miRs, in most cases, execute their functional role through post-transcriptional silencing of their target genes via direct interaction with 3' untranslated regions (3'-UTR) of the latter (Krol *et al*., 2010). The faulty expression profile of specific miRs has been shown to be associated with the development of several human diseases (Zhou *et al*., 2014; Thai *et al*., 2010). Recent studies have shown that the number of miRs is dysregulated in PCOS and regulate its pathological progression, providing information on their prognostic and therapeutic usefulness (Li *et al*, 2019). For example, miR-130b-3p has been shown to regulate the proliferation of granulosa cells by targeting SMAD4 (Bao *et al*., 2021). Similarly, miR-664a-3p has been shown to suppress ovarian granulosa cell growth in PCOS (He *et al*., 2020). MicroRNA-197-3p (miR-197-3p) has been shown to regulate ovarian malignancies such as ovarian cancer; however, its molecular role in PCOS is yet to be elucidated (Xie *et al*., 2020). Consistently, the present study was designed to reveal the role and explore the therapeutic implications of miR-197-3p in the treatment of PCOS.

MATERIALS AND METHODS

Human tissues

PCOS tissue samples and normal ovarian tissues were obtained from 15 PCOS patients and non-PCOS women hospitalized from April 2018 to March 2019 in the Reproductive Department of Wuhan KangJian Maternity and Infant Hospital, Wuhan, Hubei, China, respectively. PCOS diagnosis was made according to the revised Rotterdam 2003 criteria. The study in human tissues was approved by the host Institutional Ethics Committee. The

tissues were collected after receiving signed informed consent from all participants.

Cell lines and culturing

Human granulosa cell line KGN, along with normal SV40 ovarian cells, were purchased from ATCC, USA. Cell line culture was carried out using Dulbecco's modified Eagle Medium (DMEM Gibco, Carlsbad, CA) with 10% fetal bovine serum supplementation (FBS, Gibco) and 0.1 mg/mL streptomycin sulfate (Thermo Fisher Scientific, Carlsbad, CA) and 100 U/mL penicillin (Thermo Fisher Scientific). A humidified incubator was used to maintain the cell lines using an atmosphere of 5% CO_2/95% air at 37°C.

Transfection

Lipofectamine 3000 reagent (Invitrogen) was used to transfection KGN cells according to the user manual. Synthesized miR-197 mimics and respective miR-NC; si-CUL3 and respective si-NC and pcDNA-CUL3 and empty pcDNA3.1 vector were ordered from RiboBio Guangzhou, China.

RNA isolation, reverse transcription, and qRT-PCR

Total RNA from tissues and cells was extracted with the help of Trizol reagent (Invitrogen) according to the manufacturer's protocol. RevertAid cDNA (Thermo Fisher Scientific) synthesis kit was used for reverse transcription of extracted RNA following standard protocol. qRT-PCR was performed with the help of Power Track SYBR Green Master Mix (Thermo Fisher Scientific) to examine miR-197-3p and CUL3 transcript levels using U6 and GAPDH as respective endogenous controls. Relative expression levels were quantified using $2^{-\Delta\Delta Ct}$ method. The primers used for expression analysis were as follows: miR-197-3p; F, 5'-CACCACCTTCTCCACCCA-3' and R, 5'-GGGACTGGACTTGGAGTC-3', CUL3; F, 5'-TCGTAGACAGAGGCGCAATAA-3'and R, 5'-GGCAGTGCATCACTCGTTCT-3', U6; F, 5'-CTCGCTTCGGCAGCACA-3' and R, 5'-AACGCTTCACGAATTTGCGT-3' and GADPH; F, 5'-TGTGGGCATCAATGGATTTGG-3'and R, 5'-ACACCATGTATTCCGGGTCAAT-3'.

CCK-8 assay

The cell counting kit-8 (CCK-8) assay was executed to determine the proliferation of transfected KGN cells *in vitro*. Briefly, cells were cultured for the indicated durations in a 96-well plate using an initial inoculum of 2.5×10^4 cells/well. The wells were added with CCK-8 reagent and then after 2 h of incubation at 37°C, absorbance was recorded at 490 nm using a microplate spectrophotometer.

Analysis of cell apoptosis

Around 10^5 transfected KGN granulosa cells were propagated in 12-well plates at 37°C for 24 h. Around 25 μL of culture samples were placed on the glass slides. This was followed by their staining with a 2 μL solution of acridine orange and ethidium bromide (AO/EB). After the coverslips were placed, the slides were examined under a fluorescence microscope (Olympus).

KGN cells stably transfected with miR-197 mimics or miR-NC were propagated in 24-well plates at 37°C for 24 h. Apoptosis was studied using the Annexin V-FITC apoptosis detection kit (Sigma-Aldrich) according to the manufacturer's instructions. The Attune NxT flow cytometer (Thermo Fisher Scientific) was used for apoptosis analysis.

Western blot

Total proteins from transfected KGN cells were isolated using RIPA lysis and extraction buffer (Thermo Fisher Scientific). The sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used for resolving the extracted proteins. The separated proteins were then electrophoretically transferred to polyvinylidene fluoride membranes. 5% skim milk was used to block membranes for 2 h at room temperature. This was followed by incubation of membranes with specific primary antibodies at 4°C overnight for Bax, Bcl-2 caspase-3, CUL43. Human β-actin served as an internal reference protein. The membranes were then washed with phosphate buffered saline (PBS) and subsequently incubated with horseradish peroxidase conjugated secondary antibodies for 1.5 h at room temperature. Finally, the EasyBlot ECL Kit (Sangon Biotech, Shanghai, China) was used to detect the protein signals.

MicroRNA Target Analysis

In silico target identification for miR-197-3p was performed using the online TargetScan Huma 7.2 database (http://www.targetscan.org/vert_72/). For validation, CUL3 3'-UTR containing the wild-type (WT) or mutated (MUT) miR-197-3p binding site predicted using online bioinformatics was cloned into the pGL3 vector (Promega Corporation) downstream of the firefly luciferase reporter gene vector. Luciferase reporter vector constructs, CUL3-WT or CUL3-MUT, were co-transfected with miR-197 mimics or miR-NC into KGN cells with the help of Lipofectamine 3000 reagent (Invitrogen). Cells were cultured for 24 h at 37°C. Lastly, their relative luciferase activities were quantified using a dual luciferase reporter assay system (Promega Corporation) with reference to Renilla luciferase activity.

Statistical analysis

Experiments were performed with at least three replicates and results are given as the means ± S.D. GraphPad Prism 7.0 software was used to perform the data analysis. A two-tailed Student's t-test and one-way ANOVA were performed to assess differences between treatment groups. The statistical differences between the treatment groups were considered significant at $P<0.05$.

RESULTS

miR-197-3p is significantly repressed in PCOS

To obtain a preliminary idea of the molecular function of miR-197-3p in PCOS, qRT-PCR was used to investigate the expression levels of miR-197-3p from normal ovarian and PCOS tissue samples. The results were conclusive that miR-197-3p is significantly down-regulated ($P<0.05$) down-regulated in PCOS tissues (Fig. 1A). On average, PCOS tissues showed a 2.7-fold down-regulation of miR-197-3p relative to normal ovarian tissues. The expression of miR-197-3p was also analyzed in KGN granulose-like ovarian tumor cells and normal SV40 ovarian cells. KGN granulose cells were shown to exhibit 5.3-fold down-regulation of miR-197-3p in relation to miR-197-3p expression levels in SV40 ovarian cells (Fig. 1B).

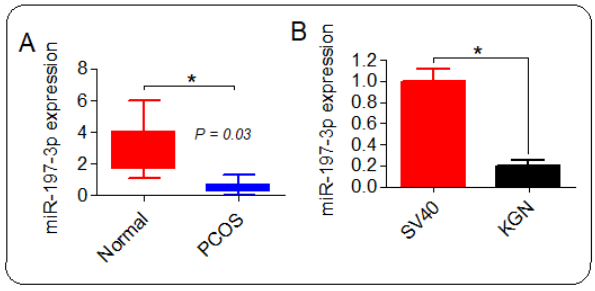

Figure 1. MiR-197-3p is down-regulated in PCOS.
Transcript levels of miR-197-3p in (**A**) normal ovarian and PCOS tissues and (**B**) KGN granulosa and normal SV40 ovarian cells. The experiments carried three biological replicates and statistical significance was assessed at *P<0.05.

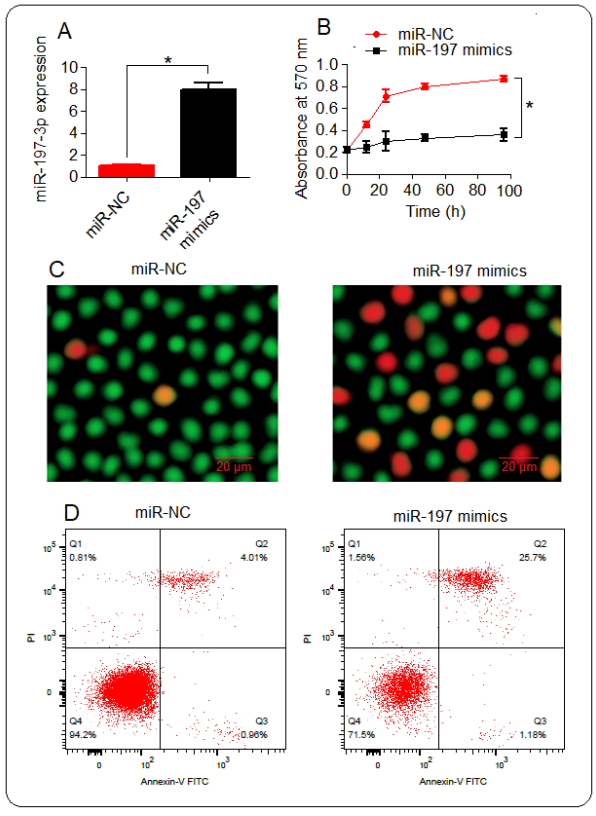

Figure 2. MiR-197-3p inhibits granulosa cell proliferation through induction of apoptosis.
(**A**) Expression of miR-197-3p in KGN granulosa cells transfected with miR-197-3p mimics or miR-NC (**B**) CCK-8 proliferation assay of KGN granulosa cells transfected with miR-197-3p mimics or miR-NC (**C**) AO/EB staining of KGN granulosa cells transfected with miR-197-3p mimics or miR-NC (green color, yellow color and red color cells depict normal, early apoptotic and late apoptotic cells, respectively) (**D**) Annexin V-FITC/PI staining of KGN granulosa cells transfected with miR-197-3p mimics or miR-NC. The experiments carried three biological replicates and statistical significance was assessed at P<0.05.

MiR-197-3p inhibits granulose cell proliferation in PCOS by inducing apoptosis

To characterize the role of miR-197-3p in PCOS, miR-197-3p was overexpressed in KGN cells by transfection with miR-197 mimics, while miR-NC transfected KGN cells were used as negative control. The

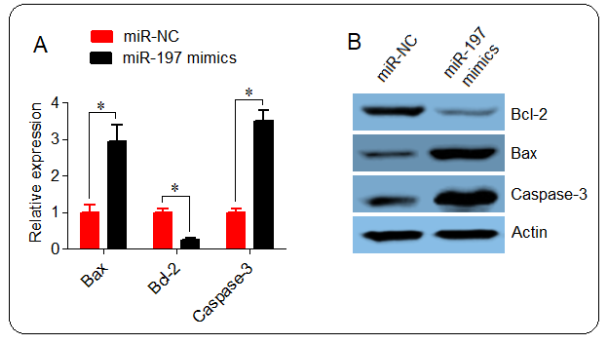

Figure 3. MiR-197-3p modulates the expression of apoptosis marker proteins.
(**A**) qRT-PCR showing the expression levels of Bax, Bcl-2 and caspase-3 in KGN granulosa cells transfected with miR-197-3p mimics or miR-NC (**B**) Western blots showing the expression levels of Bax, Bcl-2 and caspase-3 in KGN granulosa cells transfected with miR-197-3p mimics or miR-NC. The experiments carried three biological replicates and statistical significance was assessed at *P<0.05.

qRT-PCR study confirmed approximately 8 times higher miR-197-3p transcript levels in miR-197 mimics transfected cells (Fig. 2A). Subsequently, the stably transfected cells were cultured for 0, 12, 24, 48 or 96 h and the corresponding proliferation rates were analyzed at the indicated culture durations. Interestingly, miR-197-3p overexpressing KGN cells showed significantly lower (P<0.05) proliferation rates (P<0.05) compared to negative control cells (Fig. 2B). The apoptosis of transfected cells was studied to reveal the possible growth inhibitory mechanism that regulates the tumor suppressive effect of miR-197-3p overexpression. AO/EB staining revealed that overexpression of miR-197-3p triggered apoptosis in KGN cells (Fig. 2C). Induction of apoptosis in KGN cells overexpressing miR-197-3p was also verified by Annexin V-FITC/PI staining. The percentage of apoptosis was 25.7 for miR-197-3p overexpressing KGN cells as compared to only 4.01% for negative control KGN cells (Fig. 2D). qRT-PCR of Bax, Bcl-2, and caspase-3 showed that miR-197-3p overexpression significantly (P<0.05) enhanced the intracellular levels of Bax and caspase-3, while it markedly decreased Bcl-2 expression (Fig. 3A). Western blot analysis also showed an increase in Bax and capase-3 expression and a decrease in Bcl-2 expression after miR-197-3p expression (Fig. 3B). Thus, the results indicate that miR-197-3p overexpression induced apoptosis in KGN cells to limit their proliferation *in vitro*.

CUL3 is targeted by miR-197-3p in PCOS

TargetScan analysis predicted CUL3 as the regulatory target of miR-197-3p and revealed that miR-197-3p binds to a specific nucleotide site in 3'-UTR of CUL3 (Fig. 4A). The qRT-PCR expression analysis showed that CUL3 has significant up-regulation (P<0.05) in KGN cells compared to its expression in SV40 cells (Fig. 4B). The interaction of miR-197-3p with CUL3 3'-UTR was verified using a dual luciferase reporter assay. Furthermore, the targeting of CUL3 by miR-197-3p was also evidenced by the downregulation of CUL3 in KGN cells overexpressing miR-197-3p (Fig. 4C). The dual luciferase reporter assay was used to specifically verify the interaction of miR-197-3p with 3'-UTR of CUL3. The results indicated that the host KGN cells exhibited a significant decrease (P<0.05) in luciferase activity when cotransfected with mimics of

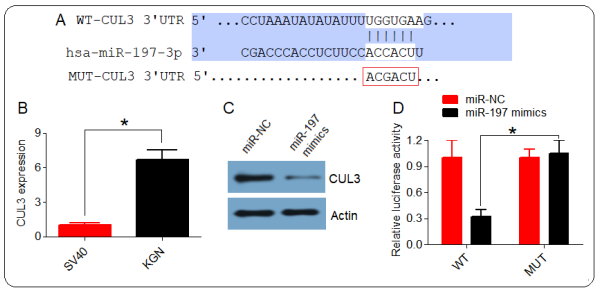

Figure 4. The miR-197-3p direct binds to 3′-UTR of CUL3.
(**A**) *In silico* target analysis of miR-197-3p and identification of its binding site in 3′-UTR of CUL3 (**B**) relative transcript levels of CUL3 in KGN granulosa and normal SV40 ovarian cells (**C**) intracellular CUL3 protein levels in (**D**) interaction analysis of miR-197-3p and CUL3 by dual luciferase reporter assay KGN granulosa cells transfected with miR-197-3p mimics or miR-NC. The experiments carried three biological replicates and statistical significance was assessed at *P<0.05.

miR-197-3p and CUL3 3'-UTR (WT) indicative of the interaction of miR-197-3p with CUL3 UTR (Fig. 4D). However, when the miR-197-3p binding site was mutated, a decrease in luciferase activity was not observed showing sequence-specific binding of miR-197-3p to 3'-UTR of CUL3.

CUL3 acts as a mediator of the molecular role of miR-197-3p in PCOS

To examine whether miR-197-3p exercises its regulatory role in PCOS by posttranscriptional silencing of CUL3, the latter was transiently down-regulated in KGN cells by si-CUL3 transfection (Fig. 5A). Next, the proliferation of KGN cells that negatively regulate miR-197-3p was assessed with reference to silencing control (si-NC) transfected cells at different culture durations. The results showed that CUL3 silencing significantly inhibited KGN cell proliferation (P<0.05) inhibited the proliferation of KGN cells, *in vitro* (Fig. 5B). On the other hand, the overexpression of CUL3 in KGN cells allowed them to proliferate at rates comparable to negative control cells even under up-regulation of miR-197-3p (Fig. 5C). Such findings are indicative that miR-197-3p executes its antiproliferative role in PCOS by silencing its target gene CUL3 at the posttranscriptional level.

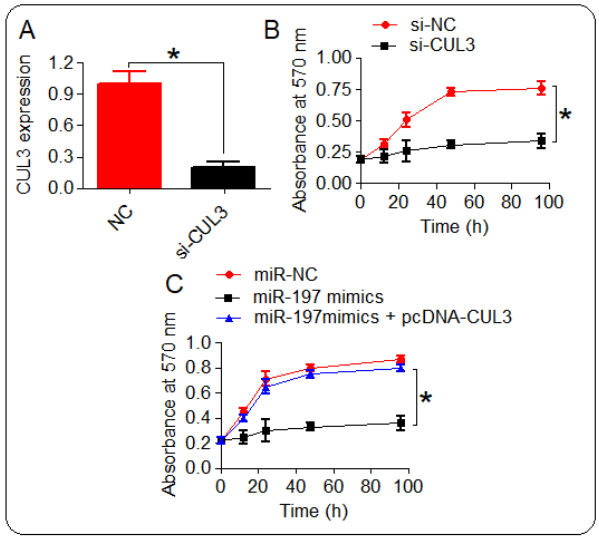

Figure 5. miR-197-3p exerts its role through CUL3 in PCOS.
(**A**) Relative transcript abundance of CUL3 in KGN cells transfected with si-CUL3 and si-NC (**B**) CCK-8 assay of KGN cells transfected with si-CUL3 and si-NC (**C**) CCK-8 assay of KGN cells transfected with miR-197 mimics, miR-197 mimics plus pcDNA-CUL3 or miR-NC. The experiments carried three biological replicates and statistical significance was assessed at *P<0.05.

DISCUSSION

Aberrant expression of microRNAs (miRs) has been reported to act as one of the prominent molecular irregularities that mediate the onset and progression of several human disorders (Maurano *et al.*, 2012). Dysregulation of miRs results in up-regulation or down-regulation of the target gene that could affect vital cell signaling pathways that lead to the development of human diseases through various modes (Hrdlickova *et al.*, 201425). The involvement of miRs in polycystic ovarian syndrome (PCOS) is well established. The miRs have been shown to regulate the pathogenesis of PCOS through multiple targets such as PTEN, WNT2B, IGF-1, and many others (He *et al.*, 2019; Jiang *et al.*, 2018, Wang *et al.*, 2019). There is growing support that miRs are actively involved in the regulation of granulosa cell growth, differentiation, and programmed death, the latter being crucial to follicular atresia limiting ovulation and thus maintaining the normal female cycle (Matsuda *et al.*, 201229). MiRs are known to exhibit both promotive and suppressive effects on the proliferation of granulosa ovarian cells (Cai *et al.*, 2017; Jiang *et al.*, 2015). The suppressive miRs are usually down-regulated in granulosa cells enabling enhanced proliferative rates. In the present study, PCOS tissues and granulosa ovarian cells showed significant down-regulation of miR-197-3p, suggesting its possible involvement in regulating the pathogenesis of PCOS. Down-regulation of miR-197-3p has also been shown to be linked with other gynecological malignancies such as breast cancer, ovarian cancer, and cervical cancer (Xu *et al.*, 2019; Hu *et al.*, 2018). Overexpression of miR-197-3p in KGN granulosa cells significantly inhibited their proliferation. The antiproliferative effects were mediated by the induction of apoptosis in granulosa cells. These findings are consistent with previous studies in which several miRs, such as miR-200b and miR-200c, have been shown to inhibit the growth of human KGN granulosa cells (He *et al.*, 2019). Studies have shown that there is remarkable interdependence between oocyte development and surrounding granulosa cells, and granulosa cell apoptosis is important for atresia of maturing follicles, particularly at the latter stages of development (Buccione *et al.*, 1990, Morita & Tilly, 1999). Furthermore, Das *et al.* have shown that enhanced granulosa cell proliferation and decreased rates of apoptosis are among the dominant molecular regularities associated with PCOS (Das *et al.*, 2008). The induction of apoptosis by miR-197-3p overexpression in granulosa cells is imperative to use miR-197-3p in the therapeutic management of PCOS, which is apparent in addition to increased levels of caspase 3, as caspase 3 has been reported to be crucial for granulosa cell apoptosis during follicular atresia (Matikainen *et al.*, 2001). The induction of apoptosis by miRs has also been previously reported, for example, miR-324 has been shown to induce apoptosis in human KGN PCOS granulosa cells (Jiang *et al.*, 2018). miR-197-3p

was shown to perform its role *via* cullin 3 (CUL3), an E3 ubiquitin ligase. Cullin 3 was shown to be highly overexpressed in granulosa ovarian cells, and its silencing inhibited the growth of KGN granulosa cells, *in vitro*. CUL3 has been reported to aid in the degradation of cell cycle inhibitory proteins and thus act as an activator of the cell cycle (Furukawa *et al.*, 2003; Luke-Glaser *et al.*, 2005). It is also known for its positive role in cytokinesis (Andérica-Romero *et al.*, 2013). In general, the results of the present study indicate that molecular targeting of CUL3 by miR-197-3p could potentially emerge as the useful therapeutic approach against PCOS in the future.

CONCLUSIONS

Taken together, the results of the present study showed that miR-197-3p is significantly down-regulated in PCOS tissues and granulosa cells. The overexpression of miR-197-3p induced apoptosis in granulosa cells by modulating caspase 3, Bax and bcl-2 protein expressions. An E3 ubiquitin ligase, CUL3, was shown to be functionally targeted by miR-197-3p to execute its molecular role in PCOS. The results point toward the potential of miR-197-3p in the management of PCOS.

Declarations

Conflict of interest. The authors declare that there are no conflicts of interest.

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