miR-613 inhibits the proliferation of human ovarian granulosa cells by arresting cell cycle progression via the targeting of IGF-1

JUNHUI WAN and SISUN LIU

Department of Obstetrics and Gynecology, The First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi 330006, P.R. China

Received May 26, 2020; Accepted October 23, 2020

DOI: 10.3892/mmr.2020.11817

Abstract. Polycystic ovary syndrome (PCOS) is a prevalent endocrine disorder, and microRNA (miRNA) molecules have been implicated in the pathological process of PCOS. The aim of the present study was to elucidate the regulatory effects of miR-613 and insulin-like growth factor-1 (IGF-1) on the pathological process of polycystic ovary syndrome (PCOS). The targeting of IGF-1 by miR-613 was investigated by Dual-luciferase reporter assay. The regulatory effect of miR-613 on the mRNA and protein levels of IGF1 was determined by reverse transcription-quantitative PCR and western blot analysis. The regulatory effects of miR-613 and IGF-1 on the proliferation and cell cycle progression of KGN cells were evaluated by colony formation assay and flow cytometric analysis. The results revealed that miR-613 targeted IGF-1 and reduced its translational level. In KGN cells, miR-613 arrested cell cycle progression in the G2/M phase and downregulated the expression of cyclin D1 and CDK1. The overexpression of IGF-1 attenuated the inhibitory effects of miR-613 on cell cycle arrest, cyclin D1 and CDK1 expression, and the proliferation of KGN cells. In conclusion, the present study demonstrated that miR-613 targets IGF-1 and thus suppresses its translation. It arrests cell cycle progression and attenuates the proliferation of KGN cells via the targeting of IGF-1. Therefore, it is suggested that miR-613 and IGF-1 could potentially be diagnostic biomarkers and therapeutic targets for PCOS.

Introduction

Polycystic ovary syndrome (PCOS) is a prevalent endocrine disorder, which is characterized by hyperandrogenaemia, chronic anovulation and polycystic ovary. PCOS is typically accompanied by insulin resistance, central obesity, cardiovascular diseases and/or early subclinical arteriosclerosis (1). It has been reported that 4-8% of women worldwide are affected by PCOS (2). Follicular dysplasia and degeneration, and thinning of the granulosa cell layer are major pathological lesions of PCOS (3), which may be attributed to increased apoptosis of granulosa cells (4). Several candidate genes have thus far been identified as pathogenic factors of PCOS, including insulin-like growth factor (IGF), insulin receptor, luteinizing hormone (LH)/human chorionic gonadotropin, sex hormone-binding globulin and DENN domain containing 1A (5-8). However, they have not been demonstrated to be the main reasons for the pathogenesis of PCOS.

MicroRNAs (miRNAs) are small-chain noncoding RNAs, 18-22 nucleotides in length, which can regulate gene expression by complementary base pairing to the 3' untranslated region (3'UTR) of target mRNAs and thus induce their degradation or inhibit translation. miRNAs are vital regulators involved in the progression of PCOS. Hossain *et al* (9) identified 89 miRNAs that are significantly upregulated or downregulated in rats with 5α-dihydrotestosterone (DHT)-induced PCOS and high levels of androgens. In blastospheres harvested from patients with PCOS, hsa-let-7a, hsa-miR-19a, hsa-miR-19b, hsa-miR-24, hsa-miR-92 and has-miR-93 were found to be significantly downregulated (10). Previous studies have shown that miR-613 has an anticancer role (11-13), but the potential function of miR-613 in the progression of PCOS remains largely unknown. The expression of miR-613 in the ovarian tissues of patients with PCOS was investigated in the present study.

The abnormal upregulation of IGF-1 and granulosa cell apoptosis are critical indicators of PCOS, whereas their potential effects are unclear. In the present study, an *in vitro* PCOS model was generated to elucidate the regulatory effects of miR-613 and IGF-1 on KGN cell phenotypes.

Materials and methods

Subjects and samples. A total of 24 patients diagnosed with PCOS in the First Affiliated Hospital of Nanchang University (Nanchang, China) were recruited from June 2018 to June 2019. During this period, 24 healthy female volunteers with matched baseline characteristics, including age, weight and waist measurement, were also recruited (Table SI). PCOS

Correspondence to: Dr Sisun Liu, Department of Obstetrics and Gynecology, The First Affiliated Hospital of Nanchang University, 17 Yongwaizheng Street, Nanchang, Jiangxi 330006, P.R. China
E-mail: liusisun@sina.com

Key words: polycystic ovary syndrome, KGN cells, microRNA-613, insulin-like growth factor-1, cell cycle progression

was diagnosed in accordance with the criteria provided in the Revised Rotterdam European Society of Human Reproduction and Embryology/American Society for Reproductive Medicine criteria (2003) (14). Ovarian tissues from the recruited subjects were collected during laparoscopic inspection or the diagnosis of pelvic pain. The study was approved by the Medical Research Ethics Committee of the First Affiliated Hospital of Nanchang University (approval no. 2018089), and written informed consent was obtained from each subject.

Cell culture. Human KGN ovarian granulosa cells and IOSE80 ovarian epithelial cells were purchased from the American Type Culture Collection (ATCC). The cells were frozen at -80˚C for storage. After recovery, the cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% foetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO_2 at 37˚C. Cell passage was conducted using trypsin until adherent cells were grown to >80% confluence.

293T cells were also purchased from the ATCC and used for lentiviral packaging. Briefly, the cells were cultured in DMEM containing 10% FBS and (both from Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO_2 at 37˚C, when the cells were >80% confluent, they were digested with trypsin and inoculated into a new culture dish. When the cells were adherent, they were incubated with serum-free Opti-MEM™ (Gibco; Thermo Fisher Scientific, Inc.) for 4 h, after which transfection and lentiviral packaging were carried out.

Cell transfection. miR-613 mimic, miR-613 inhibitor and the respective negative controls, mimic-NC and inhibitor-NC (miR-613 mimic, 5'-AGGAAUGUUCCUUCUUUGCC-3'; mimic-NC, 5'-UUCUCCGAACGUGUCACGUTT-3'; miR-613 inhibitor, 5'-GGCAAAGAAGGAACAUUCCU-3'; inhibitor-NC, 5'-UUCUCCGAACGUGUCACGUTT-3') were synthesized by Guangzhou RiboBio Co., Ltd. KGN cells were seeded at a density of $5x10^4$ cells/well in a 12-well plate and cultured for 24 h. When 80% cell confluence was reached, the medium was replaced with Opti-MEM and the cells were cultured for 4 h. The miRNAs were gently mixed with Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), then incubated for 20 min at room temperature and added to each well at a final concentration of 100 nM. The cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2 for 40 min. Opti-MEM was then replaced with DMEM containing 10% FBS and 1% penicillin-streptomycin for another 24 h of cell culture. The FAM-labeled miRNAs were observed under a fluorescence microscope to evaluate the transfection efficacy 24-48 h after transfection.

Target prediction and Dual-luciferase reporter assay. The site of IGF-1 targeted by miR-613 was predicted using TargetScan 7.2 (http://www.targetscan.org/vert_72/). Based on this, vectors containing wild-type and mutant (mut) IGF-1 3'UTRs, namely pmirGLO-IGF1-3'UTR and pmirGLO-IGF1-mut 3'UTR, were generated by CoBioer Biosciences Co., Ltd. The DNAs were directly synthesized by annealing, and the luciferase vectors were subsequently constructed using pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Corporation). In addition, an miR-613 overexpression plasmid was generated using pcDNA3.1(+) and named as pcDNA3.1(+)-miR-613. The KGN cells were cultured to 80% density and transfected with pmirGLO-IGF1-3'UTR or pmirGLO-IGF1-mut 3'UTR, alone or in combination with pcDNA3.1(+) or pcDNA3.1(+)-miR-613, using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The miRNAs and plasmids were gently mixed with Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), then incubated for 20 min at room temperature. The mRNA/plasmid/Lipofectamine 2000 mixture was then added to the cells for 40 min at 37˚C in a humidified atmosphere containing 5% CO_2. Cells were transfected at 37˚C in a humidified atmosphere containing 5% CO_2 for 48 h, and then lysed with 100 μl lysis buffer (Promega Corporation) per well and centrifuged at 14,000 x g at 4˚C for 5 min. A Dual-Luciferase Reporter Assay system (Promega Corporation) was then used to detect the luciferase activity (Promega Corporation). Relative luciferase activity was expressed as the ratio of firefly luciferase activity to *Renilla* luciferase activity.

Viability determination. Cell viability was assessed by MTT assay. Transfected cells were collected and centrifuged at 2,500 x g and 4˚C for 5 min. The precipitate was resuspended to prepare a cell suspension, which was transferred to a 96-well plate at $5x10^3$ cells/well for culture. At 24, 48, 72 and 96 h, 20 μl MTT was added to each well and incubated for 4 h at 37˚C. The cells were subsequently treated with 150 μl dimethyl sulfoxide for 10 min at room temperature to dissolve the formazan, and the optical density at 570 nm was measured using an ultraviolet spectrophotometer.

Colony formation assay. Transfected cells were seeded in 60-mm culture dishes with 100 cells/dish and then cultivated in DMEM containing 10% FBS for 14 days. Visible colonies were washed with PBS, fixed in 4% paraformaldehyde for 30 min, and stained with 1% crystal violet for 15 min at room temperature. After air-drying, images of the colonies were captured under a fluorescence microscope for counting.

Determination of cell cycle progression. Cells were transfected for 48 h, and cell cycle progression was then determined. The cells were digested with trypsin and resuspended in PBS. After fixing at 4˚C for 30 min, the cells were stained using propidium iodide (US Everbright, Inc.) at room temperature for 30 min. Cells were analyzed on a FACScan flow cytometer (BD Biosciences) and the results were analyzed using FlowJo software (FlowJo, version 10, Ashland).

Reverse transcription-quantitative PCR (RT-qPCR). RT-qPCR was conducted according to the manufacturer's protocols for the PrimeScript™ RT Reagent Kit and SYBR Premix Ex Taq™ II with Tli RNaseH (Takara Bio, Inc.), using an ABI Prism 7500 system (Thermo Fisher Scientific, Inc.). Using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), total RNA was first isolated from cells and ovarian tissues. The RNA was treated with a gDNA eraser and reversely transcribed to cDNAs. Subsequently, the cDNAs were subjected to qPCR for denaturation at 95˚C for 15 min, followed by

Table I. Primers used in the study.

Genes	Primers	Product size (bp)
miR-613	F: GTGAGTGCGTTTCCAAGTGT R: TGAGTGGCAAAGAAGGAACAT	84
IGF-1	F: CATGTCCTCCTCGCATCTCT R: AGCAGCACTCATCCACGATA	213
Cyclin D1	F: CGATGCCAACCTCCTCAACGA R: TCGCAGACCTCCAGCATCCA	153
CDK1	F: CCTAGCATCCCATGTCAAAAACTTGG R: TGATTCAGTGCCATTTTGCCAGA	108
GAPDH	F: AGCCACATCGCTCAGACAC R: GCCCAATACGACCAAATCC	66
U6	F: CTCGCTTCGGCAGCACA R: AACGCTTCACGAATTTGCGT	94

F, forward; R, reverse; miR, microRNA; IGF-1, insulin-like growth factor-1.

40 cycles of 95˚C for 5 sec, 60˚C for 30 sec and 72˚C for 40 sec. Annealing was finally conducted at 72˚C for 10 min. GAPDH and U6 were used as internal references. When analyzing changes in mRNA levels, the expression values relative to those of a housekeeping control were compared. The primer sequences and mRNA products are listed in Table I. The data were quantified using the $2^{-\Delta\Delta Ct}$ method (15).

Western blot analysis. Protein levels of IGF-1, cyclin D1 and CDK1 in cells were determined by western blot analysis. The antibodies purchased from ABclonal Biotech Co., Ltd. were as follows: Rabbit primary antibodies IGF-1 (cat. no. A11985), cyclin D1 (cat. no. A11022), CDK1 (cat. no. A0220) and GAPDH (cat. no. AC001), and HRP goat anti-rabbit secondary antibody (cat. no. AS014). Total proteins were first isolated by RIPA lysis buffer (Beyotime Institute of Biotechnology), and their concentrations were evaluated using the BCA method. Protein samples (50 μg/lane) were separated by SDS-PAGE on 12% gels, then transferred to PVDF membranes. After incubation in 5% skimmed milk for 1 h, the membranes were incubated with primary antibodies (1:1,000) at 4˚C overnight and then with secondary antibody (1:1,000) at room temperature for 1 h. The bands were visualized by Quantity One software (Bio-Rad Laboratories, Inc.).

Lentivirus transfection. Vector GV287-IGF-1 was generated by cleaving the GV287 plasmid (Shanghai GeneChem Co., Ltd.) using *Age*I and splicing in IGF-1. A 2nd generation system was used to the package of lentivirus. The lentiviral plasmid, packaging vector and envelope vector were mixed at a 4:3:2 ratio for a total DNA mass of 20 μg and incubated with 1 ml Lenti-Easy Packaging Mix (Shanghai GeneChem Co., Ltd.) for 15 min. The mixture was then incubated for another 20 min incubation with Lipofectamine® 2000, then added into 293T cell culture medium for 6 h at 37˚C. In brief, the 293T cells were seeded at a density of $2.5x10^5$ cells/plate in a 10-cm plate and cultured to 80% confluence, incubated in Opti-MEM for 4 h, and then in with the transfection mixture as described in the Cell transfection section. The supernatant of the transfected 293T cells was collected after three days by filtering through a 0.45-μm filter, and the viral particles were concentrated by ultracentrifugation at 70,000 x g for 2 h at 4˚C. The structure of the GV287 plasmid is shown in Fig. S1. KGN cells were infected with the lentivirus at a multiplicity of infection of 5 and with polybrene (Sigma-Aldrich; Merck KGaA) at a final concentration of 8 μg/ml at 37˚C with 5% CO_2 for 24 h. Fresh culture medium was then used to replace the old medium. Fluorescence was measured 72 h post-infection when the achieved infection efficiency was 80%. Screening of stable cell lines using green fluorescent protein.

Statistical analysis. Statistical analysis was performed using GraphPad 8.0 software (GraphPad Software, Inc.). Data are expressed as the mean ± standard deviation. All data in the current study conform to a normal distribution. One-way ANOVA was used to assess differences among the groups, and Tukey's and Bonferroni's tests were used for post hoc testing following ANOVA. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-613 is downregulated in patients with PCOS. To explore the biological function of miR-613 in PCOS, the levels of miR-613 in ovarian tissues and KGN cells were first examined. The results revealed that miR-613 was significantly downregulated in the ovarian tissues of patients with PCOS compared with those in the healthy controls, and in KGN cells compared with IOSE80 cells ($P<0.001$; Fig. 1A), suggesting a significant involvement of miR-613 in PCOS.

miR-613 inhibits IGF-1 expression by directly binding to its 3'UTR. Using TargetScan, binding sites were predicted for miR-613 in the 3'UTR of IGF-1 (Fig. 2A). A Dual-luciferase reporter assay then revealed that the luciferase intensity in the pmirGLO-IGF1-3'UTR + pcDNA3.1(+)-miR-613 group

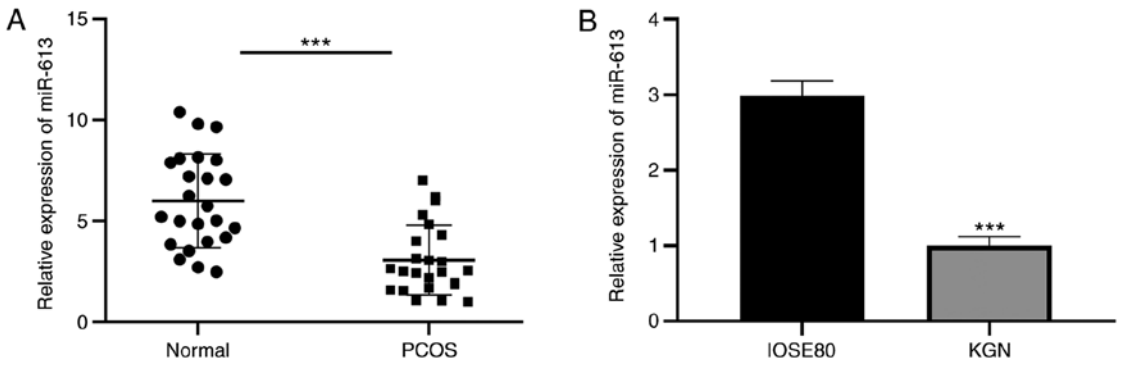

Figure 1. miR-613 is downregulated in PCOS. (A) miR-613 levels in patients with PCOS and healthy females and (B) in KGN ovarian granulosa and IOSE80 ovarian epithelial cells. ***P<0.001. miR, microRNA; PCOS, polycystic ovary syndrome.

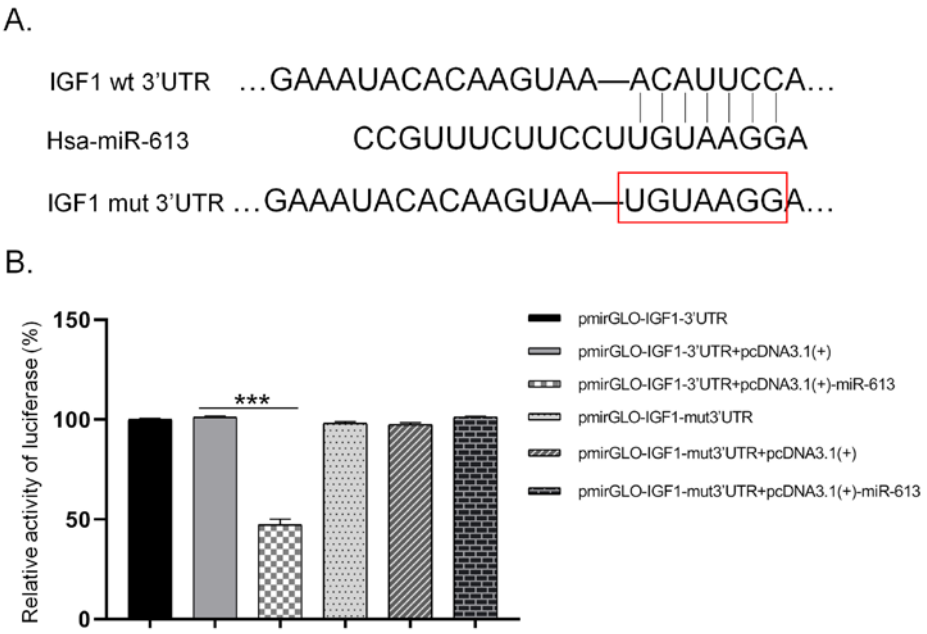

Figure 2. miR-613 inhibits IGF-1 expression by directly binding to its 3'UTR. (A) Binding sites predicted using TargetScan; (B) Dual-luciferase reporter assay results confirming the binding between miR-613 and IGF-1. ***P<0.001. miR, microRNA; IGF-1, insulin-like growth factor-1; 3'UTR, 3' untranslated region.

was only about 47% of that in the pmirGLO-IGF1-3'UTR + pcDNA3.1(+) group, which was a significant difference (P<0.001). Furthermore, no significant difference in luciferase intensity was observed between the pmirGLO-IGF1-mut 3'UTR + pcDNA3.1(+)-miR-613 group and the pmirGLO-IGF1-mut 3'UTR + pcDNA3.1(+) group (Fig. 2B). These results demonstrate that miR-613 targeted the 3'UTR of IGF-1 and thus regulated its post-transcriptional level.

Overexpression of miR-613 inhibits KGN cell proliferation. Total RNAs and proteins were extracted from KGN cells transfected with miR-613 mimic, miR-613 inhibitor or the respective negative controls and then subjected to RT-qPCR and western blot analysis, respectively (Fig. 3A and B). Transfection of the KGN cells with miR-613 mimic markedly reduced the translational level of IGF-1. MTT assay revealed that transfection with miR-613 mimic significantly decreased the viability of KGN cells, whereas the knockdown of miR-613 significantly increased cell KGN cell viability compared with the respective negative controls (P<0.01; Fig. 3C). Colony formation was reduced in KGN cells overexpressing miR-613, whereas the knockdown of miR-613 increased the number of colonies formed, compared with the respective negative controls (P<0.05; Fig. 3D). Collectively, these results indicate that miR-613 inhibited KGN cell proliferation.

Overexpression of miR-613 inhibits KGN cell proliferation by arresting cell cycle progression. Flow cytometry was used to assess the effect of abnormal expression levels of miR-613 on cell cycle progression in KGN cells. Compared with the respective control, the KGN cells overexpressing miR-613 showed a reduced percentage of cells in the S phase and an increased percentage of cells in the G2/M phase, whereas the knockdown of miR-613 yielded the opposite results (P<0.05; Fig. 4 and B). Moreover, whether the expression of levels of cell cycle-associated mRNAs and proteins were regulated by miR-613 was determined by RT-qPCR and western blot analysis, respectively. The overexpression of miR-613 down-

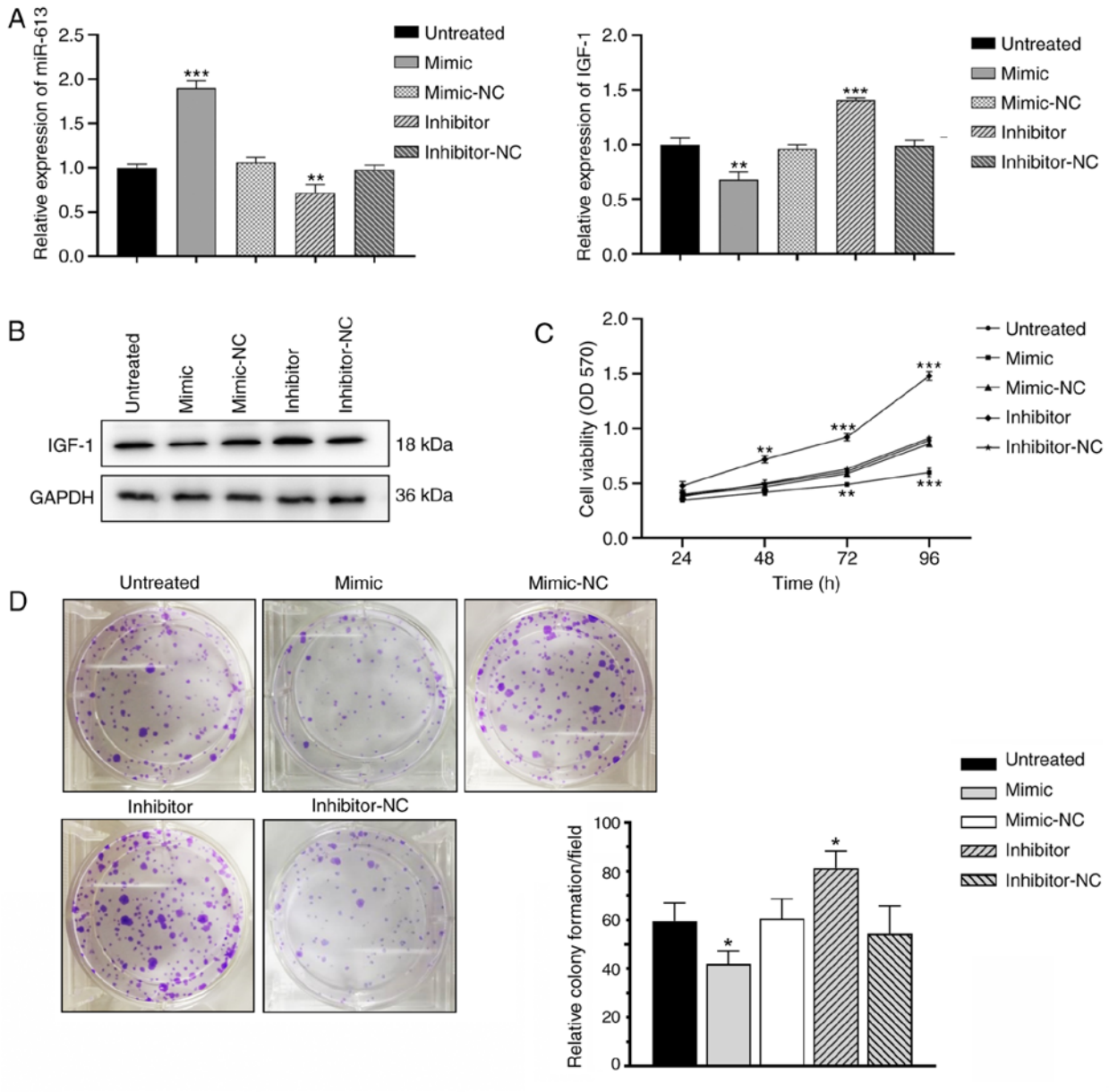

Figure 3. Overexpression of miR-613 reduces KGN cell proliferation. (A) miR-613 and IGF-1 mRNA levels in transfected KGN cells. (B) IGF-1 levels in transfected KGN cells detected using western blotting. (C) Viability of KGN cells is regulated by miR-613. (D) Number of colonies formed by KGN cells is regulated by miR-613. *P<0.05, **P<0.01, ***P<0.001, vs. untreated group. miR, microRNA; IGF-1, insulin-like growth factor-1; OD 570, optical density at 570 nm; mimic, miR-613 mimic; inhibitor, miR-613 inhibitor; NC, negative control.

regulated the expression of cyclin D1 and CDK1, whereas the knockdown of miR-613 upregulated them at the mRNA (P<0.05) and protein levels (Fig. 4C and D). These results indicate that the overexpression of miR-613 arrested cell cycle progression in the G2/M phase by downregulating cyclin D1 and CDK1, and inhibited KGN cell proliferation.

Overexpression of IGF-1 promotes KGN cell proliferation. The KGN cell line with stably overexpressed IGF-1 was generated by lentiviral transfection. IGF-1 expression was significanty increased following lentivirus infection, compared with cells transduce with an empty vector (Fig. S2). Subsequently, miR-613 mimic, miR-613 inhibitor, or their negative controls were transfected. The mRNA and protein levels of IGF-1 were assessed. Overexpression of IGF-1 significantly reversed the effects of miR-613 mimics and increased the expression of IGF-1 (Fig. 5A and B). Using MTT and colony formation assays, the overexpression of IGF-1 was shown to markedly increase the viability and proliferation of KGN cells compared with that in the respective control (Fig. 5C and D).

Overexpression of IGF-1 accelerates cell cycle progression in KGN cells. The regulatory effect of IGF-1 overexpression on the cell cycle progression of KGN cells was evaluated. Compared with that in the respective control, the cell cycle arrest in KGN cells induced by the overexpression of miR-613 was significantly attenuated by the overexpression of IGF-1, and the overexpression of IGF-1 further accelerated cell cycle progression in KGN cells with miR-613 knockdown (P<0.05; Fig. 6A and B). Moreover, the overexpression of IGF-1 elimi-

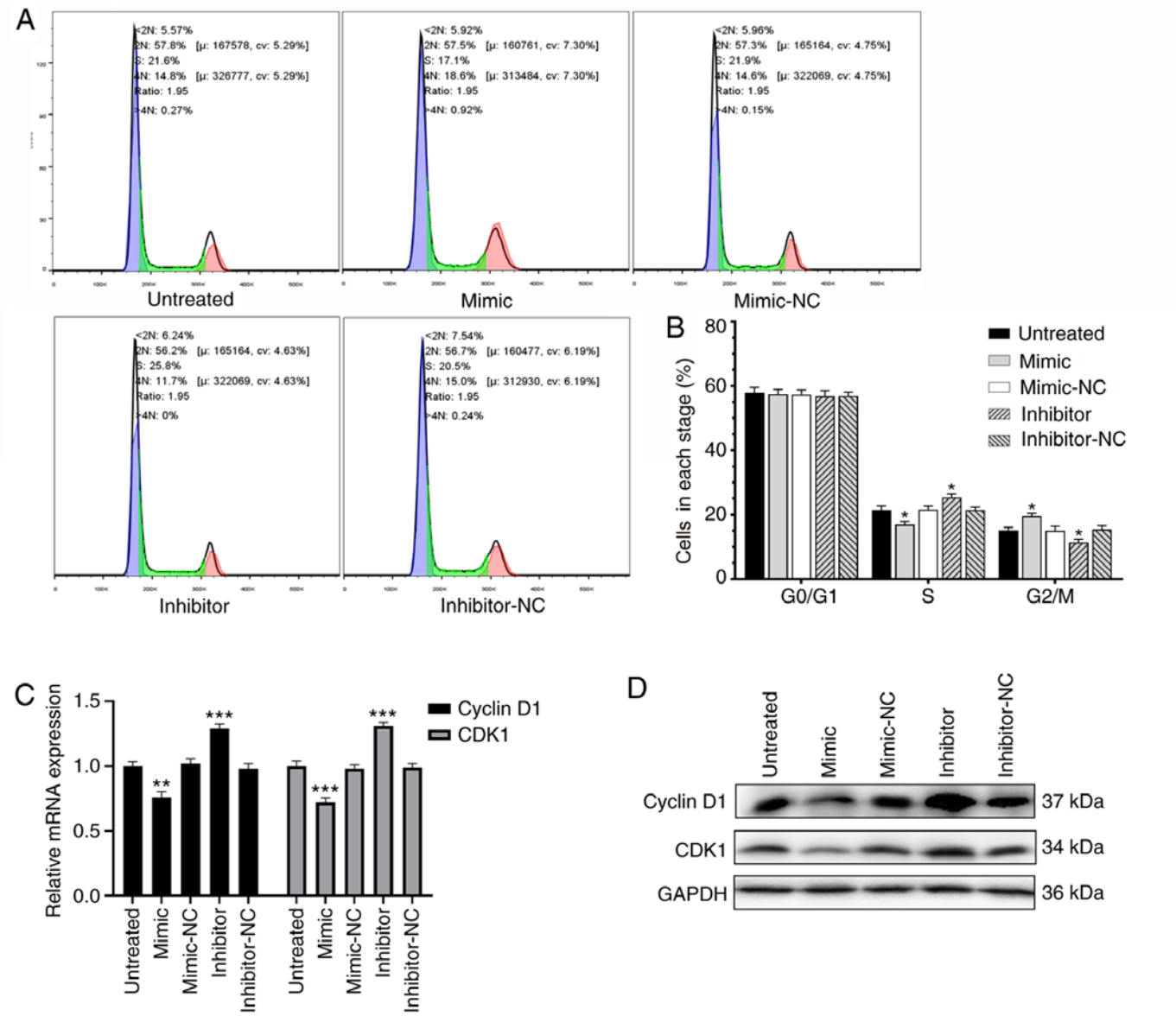

Figure 4. Overexpression of miR-613 arrests cell cycle progression. (A) Representative flow cytometry plots and (B) histogram showing the cell cycle distribution in transfected KGN cells. (C) Relative mRNA levels of cyclin D1 and CDK1. (D) Protein levels of cyclin D1 and CDK1 detected using western blotting. *P<0.05, **P<0.01, ***P<0.001,vs. untreated group. miR, microRNA; mimic, miR-613 mimic; inhibitor, miR-613 inhibitor; NC, negative control.

nated the inhibitory effects of miR-613 on the expression of cyclin D1 and CDK1 at the mRNA (P<0.01) and protein levels (Fig. 6C and D). These results indicate that the inhibitory effects of miR-613 on the proliferation and cell cycle progression of KGN cells may be attributable to IGF-1.

Discussion

PCOS is a common endocrine and metabolic disease in women of reproductive age. The disease has a complex etiology, and its pathogenesis remains largely unclear. Pathological changes that occur in PCOS include the presence of multiple immature follicles in the ovary, lack of dominant follicle selection, atresia of small follicles, and degeneration and thinning of the granular cell layer (3). Follicular growth and development are delicate and complex processes in which granulosa cells are of great significance. PCOS-induced elevated androgen levels, insulin resistance and chronic inflammation have a close association with granulosa cells (16). High androgen levels are the most typical hormonal change observed in patients with PCOS, and are associated with hair thinning, anovulation, infertility, hirsutism, acne and seborrheic alopecia (16). Yang *et al* (17) observed an increase in the level of testosterone in follicular fluid and a reduction in aromatase expression in the luteinized granulosa cells of patients with PCOS, indicating an association between high androgen levels in follicles and the progression of PCOS. Insulin resistance and compensatory hyperinsulinemia are important characteristics of PCOS, which may result in metabolic syndrome, impaired glucose tolerance and reproductive disorders. Belani *et al* (18) determined that high levels of insulin stimulate apoptosis in the granulosa cells of rats; however, the molecular mechanism underlying this has not yet been identified. Moreover, patients with PCOS exist in a pathological state of chronic and low-level

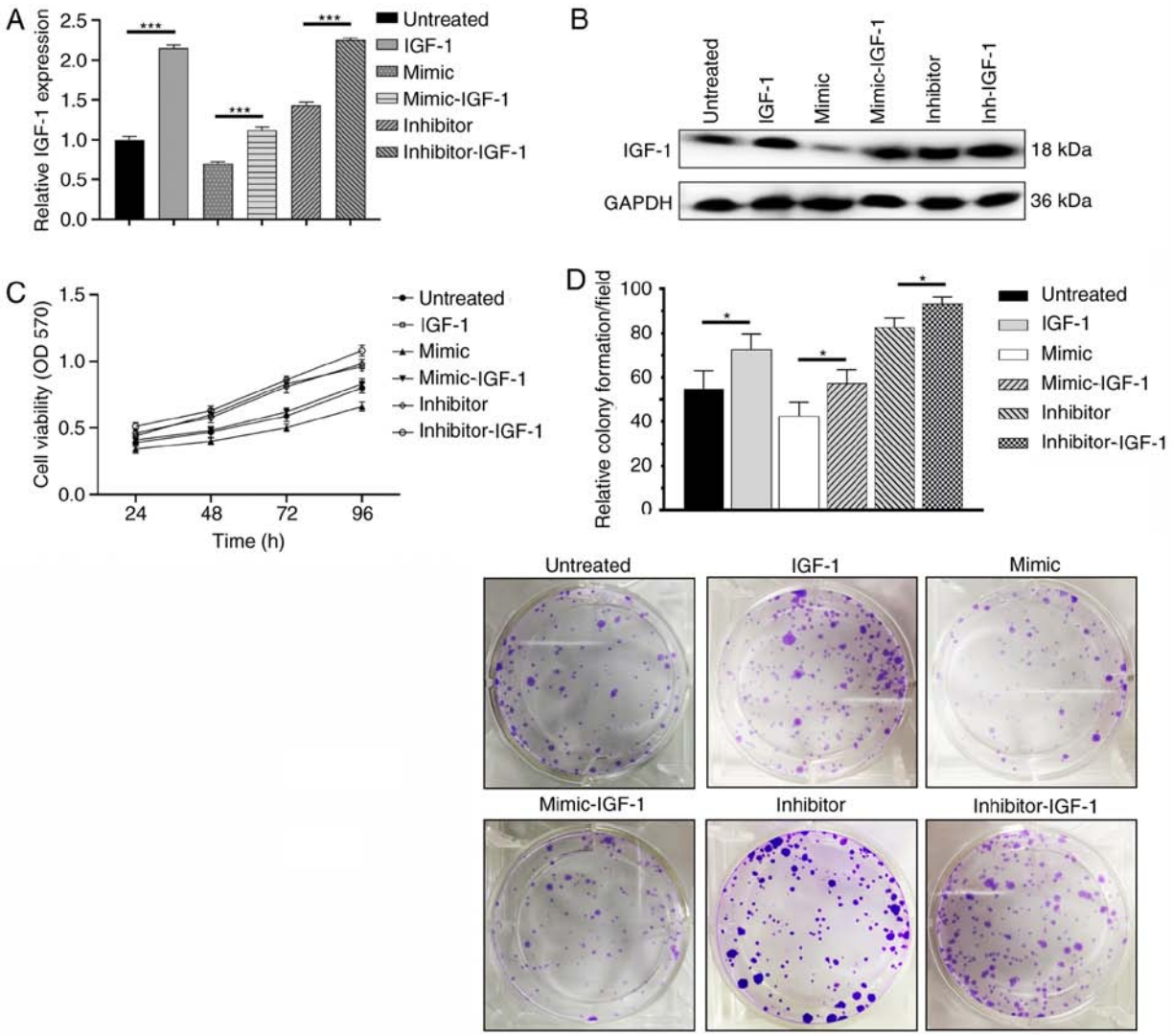

Figure 5. Overexpression of IGF-1 promotes KGN cell proliferation. (A) Relative levels of IGF-1 mRNA are regulated by miR-613. (B) Protein levels of IGF-1 detected using western blotting. (C) Viability of KGN cells is regulated by miR-613 and IGF-1. (D) Number of colonies formed by KGN cells is regulated by miR-613 and IGF-1. *P<0.05, ***P<0.001. IGF-1, insulin-like growth factor-1; miR, microRNA; NC, negative control; OD 570, optical density at 570 nm.

inflammation. The upregulation of inflammation-associated genes in the granulosa cells of patients with PCOS may be attributed to abnormal ovulation and ovarian hyperstimulation syndrome. A recent study proposed that high levels of androgen activate ovarian chemerin and thereby stimulate the chemokine-induced recruitment of monocytes to the ovaries (19). Zhang *et al* (20) detected high expression levels of lysyl oxidase and interleukin-1β in the granulosa cells and follicular fluid of patients with PCOS. They also found that the inhibition of lysyl oxidase activity ameliorated anovulation, suggesting that anti-inflammatory treatment is effective for PCOS. Granulosa cells are generally regarded as being vital during follicular development and the pathological process of PCOS.

miRNAs are endogenous, small-molecular, non-coding RNAs that induce the degradation, inhibit translation or promote the deadenylation of target mRNAs by recognizing and binding to the 3'UTR of target genes. miRNAs are extensively involved in the pathological process of PCOS. Sirotkin *et al* (21) reported that 36 of 80 different miRNAs tested in human ovarian cells exerted regulatory effects on progesterone secretion, among which, notably, miR-107 significantly stimulated androgen secretion. Yao *et al* (22) demonstrated the involvement of miR-224 in the regulation of the TGF-β1-induced proliferation of mouse granulosa cells and the stimulation of estrogen secretion, which proceeded via the targeting of Smad4. In addition, miR-224 has been reported to be highly expressed in the follicular fluid of patients with PCOS, indicating a potential role of miR-224 in the hormonal regulation of PCOS (23). Furthermore, in a DTH-induced rat model of PCOS, 17 downregulated miRNAs and 27 upregulated miRNAs were identified in rats with hyperandrogenemia (9). It has been suggested that the expression of miRNAs in patients with PCOS may be affected by obesity and the serum level of free testosterone (24).

The first study to feature miR-613 was reported in 2011 (25). miR-613 has been identified to have anticancer effects. For example, Wang *et al* (11) identified that miR-613 is significantly downregulated in human cytomegalovirus-positive glioblastoma, and plays a tumor-suppressive role

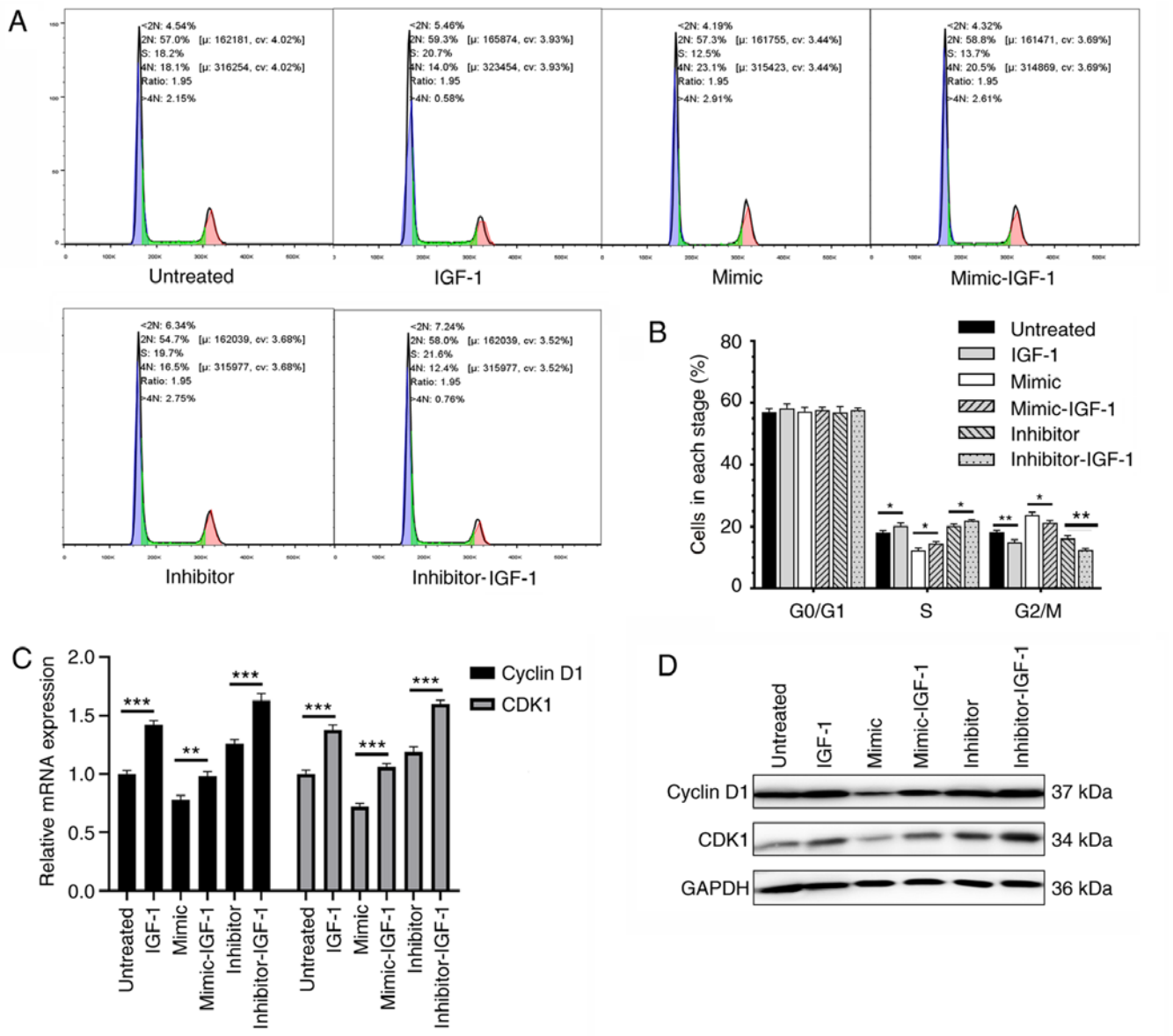

Figure 6. Overexpression of IGF-1 accelerates cell cycle progression in KGN cells. (A) Representative flow cytometry plots and (B) histogram showing the cell cycle distribution in transfected KGN cells. (C) Relative mRNA levels of cyclin D1 and CDK1. (D) Protein levels of cyclin D1 and CDK1 detected using western blotting. *P<0.05, **P<0.01, ***P<0.001. IGF-1, insulin-like growth factor-1; NC, negative control.

via the targeting of arginase 2. Another study demonstrated that as gastric cancer progresses, miR-613 arrests cell cycle progression by targeting the cell cycle protein CDK9 (26). Furthermore, miR-613 has been demonstrated to attenuate the proliferation and invasion of glioma cells by targeting CDK14 (12). Gao *et al* (27) revealed that miR-613 prevents angiogenesis in nasopharyngeal carcinoma by inactivation of the Akt signaling pathway via FN1 downregulation, thereby attenuating tumor progression. In addition, the progression and metastasis of colorectal carcinoma have been shown to be inhibited by miR-613-mediated cell cycle arrest in the G1 phase, with miR-613 directly targeting FMNL2 (13). However, the role of miR-613 in the regulation of granulosa cells and PCOS progression has rarely been reported. The findings of the present study demonstrate that miR-613 was downregulated in the ovarian tissues of PCOS patients and in KGN cells. In addition, miR-613 regulated the transcription activity of IGF-1 via directly targeting its 3'UTR, thereby arresting cell cycle progression and inhibiting the proliferation of KGN cells.

IGF-1 regulates cell growth and proliferation, which serve critical roles in tumor development (28). Wang *et al* (29) demonstrated that blocking autocrine IGF-1 via the application of a specific anti-IGF-1 antibody inactivated the Akt/GSK-3β signaling pathway, which contributed to the reduced activity of umbilical cord mesenchymal stem cells and the arrest of cell cycle progression. IGF-1 is closely associated with hyperinsulinemia and mammalian aging. Furthermore, in worms, insects and yeast, the inhibition of insulin/IGF-like signal transduction prolongs their lifespans (30-32). Anisimov (33) and Bartke (34) reported that IGF-1 deficiency significantly extends the lifespan of mice. Mice with IGF-1 deficiency exhibit decreased IGF-1 and insulin levels in the circulation but markedly increased insulin resistance. Previous studies have detected high

expression levels of IGF-1 in the ovarian tissues and granulosa cells of patients with PCOS. In one of these studies, IGF-1 was found to be upregulated in the cumulus cells collected from patients with PCOS, whereas miR-483-5p and miR-486-5p were downregulated. Further analysis suggested that miR-483-5p significantly regulates insulin resistance, and miR-486-5p triggers cumulus cell proliferation by activating the PI3K/Akt signaling pathway (35). In another study, Homburg *et al* (36) collected serum samples from patients with PCOS or hypopituitarism, and healthy subjects to detect the levels of IGF-1, IGF binding protein-1, insulin and LH. Patients with PCOS were found to express markedly high levels of IGF-1, insulin and LH. In the current study, IGF-1 was upregulated in the ovarian tissues of patients with PCOS and in KGN cells. The translation of IGF-1 was inhibited by miR-613, which downregulated the expression of IGF-1 in KGN cells. The reduced expression of IGF-1 was associated with changes in the expression of cyclin D1 and CDK1 at the protein level, arrest at the G2/M phase of the cell cycle and the inhibition of KGN cell proliferation.

In conclusion, miR-613 is significantly downregulated in the ovarian tissues of patients with PCOS and in KGN cells. It arrests cell cycle progression and attenuates the proliferation of KGN cells via the targeting of IGF-1. Therefore, miR-613 and IGF-1 may be potential diagnostic biomarkers and therapeutic targets for PCOS.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Key R&D Program Projects in Jiangxi Province (grant no. 20171BBG70009).

Availability of data and materials

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

Authors' contributions

JW and SL made substantial contributions to conception and design, acquisition, analysis and interpretation of data, and given final approval of the version to be published together. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved. SL drafted the manuscript and revised it critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Medical Research Ethics Committee of the First Affiliated Hospital of Nanchang University (approval no. 2018089), and written informed consent was obtained from each subject.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Pepene CE, Ilie IR, Marian I and Duncea I: Circulating osteoprotegerin and soluble receptor activator of nuclear factor κB ligand in polycystic ovary syndrome: Relationships to insulin resistance and endothelial dysfunction. Eur J Endocrinol 164: 61-68, 2011.
2. Franik S, Eltrop SM, Kremer JA, Kiesel L and Farquhar C: Aromatase inhibitors (letrozole) for subfertile women with polycystic ovary syndrome. Cochrane Database Syst Rev 5: CD010287, 2018.
3. Broekmans FJ and Fauser BC: Diagnostic criteria for polycystic ovarian syndrome. Endocrine 30: 3-11, 2006.
4. Ding L, Gao F, Zhang M, Yan W, Tang R, Zhang C and Chen ZJ: Higher PDCD4 expression is associated with obesity, insulin resistance, lipid metabolism disorders, and granulosa cell apoptosis in polycystic ovary syndrome. Fertil Steril 105: 1330-1337.e3, 2016.
5. Kosova G and Urbanek M: Genetics of the polycystic ovary syndrome. Mol Cell Endocrinol 373: 29-38, 2013.
6. Urbanek M: The genetics of the polycystic ovary syndrome. Nat Clin Pract Endocrinol Metab 3: 103-111, 2007.
7. Chen ZJ, Zhao H, He L, Shi Y, Qin Y, Shi Y, Li Z, You L, Zhao J, Liu J, *et al*: Genome-wide association study identifies susceptibility loci for polycystic ovary syndrome on chromosome 2p16.3, 2p21 and 9q33.3. Nat Genet 43: 55-59, 2011.
8. Diamanti-Kandarakis E and Piperi C: Genetics of polycystic ovary syndrome: Searching for the way out of the labyrinth. Hum Reprod Update 11: 631-643, 2005.
9. Hossain MM, Cao M, Wang Q, Kim JY, Schellander K, Tesfaye D and Tsang BK: Altered expression of miRNAs in a dihydrotestosterone-induced rat PCOS model. J Ovarian Res 6: 36, 2013.
10. McCallie B, Schoolcraft WB and Katz-Jaffe MG: Aberration of blastocyst microRNA expression is associated with human infertility. Fertil Steril 93: 2374-2382, 2010.
11. Wang Y, Zhao P, Qian D, Hu M, Zhang L, Shi H and Wang B: MicroRNA-613 is downregulated in HCMV-positive glioblastoma and inhibits tumour progression by targeting arginase-2. Tumour Biol 39: 1010428317712512, 2017.
12. Li Q, Zhou L, Wang M, Wang N, Li C, Wang J and Qi L: MicroRNA-613 impedes the proliferation and invasion of glioma cells by targeting cyclin-dependent kinase 14. Biomed Pharmacother 98: 636-642, 2018.
13. Li B, Xie Z, Li Z, Chen S and Li B: MicroRNA-613 targets FMNL2 and suppresses progression of colorectal cancer. Am J Transl Res 8: 5475-5484, 2016.
14. Kanafchian M, Esmaeilzadeh S, Mahjoub S, Rahsepar M and Ghasemi M: Status of serum copper, magnesium, and total antioxidant capacity in patients with polycystic ovary syndrome. Biol Trace Elem Res 193: 111-117, 2020.
15. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.
16. Rosenfield RL and Ehrmann DA: The pathogenesis of polycystic ovary syndrome (PCOS): The hypothesis of PCOS as functional ovarian hyperandrogenism revisited. Endocr Rev 37: 467-520, 2016.
17. Yang F, Ruan YC, Yang YJ, Wang K, Liang SS, Han YB, Teng XM and Yang JZ: Follicular hyperandrogenism downregulates aromatase in luteinized granulosa cells in polycystic ovary syndrome women. Reproduction 150: 289-296, 2015.
18. Belani M, Deo A, Shah P, Banker M, Singal P and Gupta S: Differential insulin and steroidogenic signaling in insulin resistant and non-insulin resistant human luteinized granulosa cells-A study in PCOS patients. J Steroid Biochem Mol Biol 178: 283-292, 2018.

19. Lima PDA, Nivet AL, Wang Q, Chen YA, Leader A, Cheung A, Tzeng CR and Tsang BK: Polycystic ovary syndrome: Possible involvement of androgen-induced, chemerin-mediated ovarian recruitment of monocytes/macrophages. Biol Reprod 99: 838-852, 2018.
20. Zhang C, Ma J, Wang W, Sun Y and Sun K: Lysyl oxidase blockade ameliorates anovulation in polycystic ovary syndrome. Hum Reprod 33: 2096-2106, 2018.
21. Sirotkin AV, Lauková M, Ovcharenko D, Brenaut P and Mlyncek M: Identification of microRNAs controlling human ovarian cell proliferation and apoptosis. J Cell Physiol 223: 49-56, 2010.
22. Yao G, Yin M, Lian J, Tian H, Liu L, Li X and Sun F: MicroRNA-224 is involved in transforming growth factor-beta-mediated mouse granulosa cell proliferation and granulosa cell function by targeting Smad4. Mol Endocrinol 24: 540-551, 2010.
23. Roth LW, McCallie B, Alvero R, Schoolcraft WB, Minjarez D and Katz-Jaffe MG: Altered microRNA and gene expression in the follicular fluid of women with polycystic ovary syndrome. J Assist Reprod Genet 31: 355-362, 2014.
24. Murri M, Insenser M, Fernández-Durán E, San-Millán JL and Escobar-Morreale HF: Effects of polycystic ovary syndrome (PCOS), sex hormones, and obesity on circulating miRNA-21, miRNA-27b, miRNA-103, and miRNA-155 expression. J Clin Endocrinol Metab 98: E1835-E1844, 2013.
25. Ou Z, Wada T, Gramignoli R, Li S, Strom SC, Huang M and Xie W: MicroRNA hsa-miR-613 targets the human LXRα gene and mediates a feedback loop of LXRα autoregulation. Mol Endocrinol 25: 584-596, 2011.
26. Lu Y, Tang L, Zhang Q, Zhang Z and Wei W: MicroRNA-613 inhibits the progression of gastric cancer by targeting CDK9. Artif Cells Nanomed Biotechnol 46: 980-984, 2018.
27. Gao R, Feng Q and Tan G: microRNA-613 exerts anti-angiogenic effect on nasopharyngeal carcinoma cells through inactivating the AKT signaling pathway by down-regulating FN1. Biosci Rep 39: BSR20182196, 2019. doi: 10.1042/BSR20182196.
28. Clemmons DR: Modifying IGF1 activity: An approach to treat endocrine disorders, atherosclerosis and cancer. Nat Rev Drug Discov 6: 821-833, 2007.
29. Wang Q, Zhang F and Hong Y: Blocking of autocrine IGF-1 reduces viability of human umbilical cord mesenchymal stem cells via inhibition of the Akt/Gsk-3β signaling pathway. Mol Med Rep 17: 4681-4687, 2018.
30. Guarente L and Kenyon C: Genetic pathways that regulate ageing in model organisms. Nature 408: 255-262, 2000.
31. Longo VD and Finch CE: Evolutionary medicine: From dwarf model systems to healthy centenarians? Science 299: 1342-1346, 2003.
32. Tatar M, Bartke A and Antebi A: The endocrine regulation of aging by insulin-like signals. Science 299: 1346-1351, 2003.
33. Anisimov VN and Bartke A: The key role of growth hormone-insulin-IGF-1 signaling in aging and cancer. Crit Rev Oncol Hematol 87: 201-223, 2013.
34. Bartke A: Single-gene mutations and healthy ageing in mammals. Philos Trans R Soc Lond B Biol Sci 366: 28-34, 2011.
35. Shi L, Liu S, Zhao W and Shi J: miR-483-5p and miR-486-5p are down-regulated in cumulus cells of metaphase II oocytes from women with polycystic ovary syndrome. Reprod Biomed Online 31: 565-572, 2015.
36. Homburg R, Pariente C, Lunenfeld B and Jacobs HS: The role of insulin-like growth factor-1 (IGF-1) and IGF binding protein-1 (IGFBP-1) in the pathogenesis of polycystic ovary syndrome. Hum Reprod 7: 1379-1383, 1992.