Long non-coding RNA LINC-01572:28 inhibits granulosa cell growth via a decrease in p27 (Kip1) degradation in patients with polycystic ovary syndrome

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Abstract

Background: Disordered folliculogenesis is a key feature of polycystic ovary syndrome (PCOS), but the underlying molecular mechanism remains unclear.

Methods: Long non-coding RNA (lncRNA) expression in luteinized granulosa cells (hLGCs) derived from women with and without PCOS were analyzed using microarray and qRT-PCR. Immortalized human granulosa cell lines were cultured for proliferation assays after transfection with the LINC-01572:28 over-expression vector in the presence or absence of p27 siRNA. Protein expression analysis, rescue assays, and RNA immunoprecipitation (RIP) were used to confirm the LINC-01572:28 substrate.

Findings: LINC-01572:28 and p27 protein were elevated whereas proliferating cell nucleolin antigen protein was decreased in the hLGCs of women with PCOS. LINC-01572:28 expression was positively correlated with basal testosterone levels. Over-expression of LINC-01572:28 inhibited cell proliferation and impeded G1/S transition, which were partially reversed by siRNA-mediated p27 knockdown.

Interpretation: Our findings, therefore, suggest that LINC-01572:28 suppresses cell proliferation and cell cycle progression by reducing the degradation of p27 protein via SKP2 binding.

1. Introduction

Polycystic ovary syndrome (PCOS), a common endocrine disease among reproductive-aged women [21], is characterized by hyperandrogenemia, chronic oligo/anovulation, and polycystic ovarian morphology [7,35]. PCOS is a leading cause of female infertility. Although women with PCOS exhibit more follicles than women without PCOS [11], none of these follicles develop into a dominant follicle, leading to abnormal ovulation. The granulosa cell layers surrounding these follicles show signs of atresia, degradation, and hypertrophy, indicating abnormal proliferation and/or apoptosis [1]. The granulosa cells are essential for providing the oocyte with nutrients and growth regulators during oocyte development [22,25]. Their dysfunction, therefore, may contribute to the aberrant folliculogenesis observed in PCOS.

Microarray analysis of tissue from women with and without PCOS identifies a significant proportion of the differentially expressed transcripts in PCOS as non-coding RNAs. Non-coding RNA, especially long non-coding RNA (lncRNA), have important potential regulatory effects on gene expression. lncRNAs, which are defined as transcripts longer than 200 nucleotides without coding potential [33], play a crucial role in cell development, differentiation, proliferation, and apoptosis via interactions with RNA-binding proteins, chromatin modification, and ceRNA networks [6,32]. Previous studies have demonstrated that lncRNAs may be involved in follicular development. For example, Yerushalmi et al. found that lncRNA Neat1 knockout (KO) mice were unable to become pregnant due to corpus luteum dysfunction and low progesterone levels [27]. Furthermore, Huang et al. demonstrated different microarray expression patterns of lncRNAs and mRNAs in cumulus cells isolated from patients with and without PCOS [10]. Liu et al. also found differential expression of lncRNA-HGC26 in women with PCOS, which may influence the proliferation and steroidogenesis of the granulosa cells [20]. Despite these findings, however, the molecular...
Research in context

Evidence before this study

Polycystic ovary syndrome (PCOS), is a leading cause of female infertility. Although women with PCOS exhibit more follicles than women without PCOS, none of these follicles develop into a dominant follicle, leading to abnormal ovulation. The granulosa cell layers surrounding these follicles show signs of atresia, degradation, and hypertrophy, indicating abnormal proliferation and/or apoptosis. The granulosa cells are essential for providing the oocyte with nutrients and growth regulators during oocyte development. Their dysfunction, therefore, may contribute to the aberrant folliculogenesis observed in PCOS.

Previous studies have demonstrated that IncRNAs may be involved in follicular development. For example, Yerushalmi et al. found that IncRNA Neat1 knockout (KO) mice were unable to become pregnant due to corpus luteum dysfunction and low progesterone levels. Furthermore, Huang et al. demonstrated different microarray expression patterns of IncRNAs and mRNAs in cumulus cells isolated from patients with and without PCOS. Liu et al. also found differential expression of IncRNA-HCG26 in women with PCOS, which may influence the proliferation and steroidogenesis of the granulosa cells. Despite these findings, however, the molecular mechanism underlying the involvement of IncRNAs in disordered folliculogenesis in PCOS remains unclear.

In this study, we conducted microarray analysis to identify differentially expressed protein-coding genes and IncRNAs expression profiles in luteinized granulosa cells obtained from women with and without PCOS. Diagnosis of PCOS was based on the following revised Rotterdam diagnostic criteria for PCOS [35], which requires the presence of at least 2 of the following criteria for a PCOS diagnosis: (i) oligo-ovulation and/or anovulation; (ii) clinical and/or biochemical signs of hyperandrogenism; and (iii) polycystic ovaries. Diagnoses of PCOS were made after exclusion of other etiologies for hyperandrogenemia and ovulatory dysfunction (eg. 21-hydroxylase deficiency, congenital adrenal hyperplasia, Cush- ing syndrome, androgen-secreting tumors, thyroid disease, and hyperprolactinemia). All subjects in the control group had regular menstrual cycles (26–35 days) and normal ovarian morphology; total testosterone was evaluated to exclude hyperandrogenism. Peripheral blood samples were collected from all subjects during days 2 to 4 of spontaneous cycles or after cessation of bleeding after a 12-h overnight fast.

Added value of this study

In the current study, we demonstrated that the abundance of LINC-01572:28 was elevated in the hLGCs of women with PCOS compared to those from control women using microarray and qRT-PCR. The elevation of LINC-01572:28 impeded the interaction between SKP2 and p27 via competitive binding to SKP2, which resulted in the accumulation of p27. The accumulation of p27 in turn induced cell cycle arrest and inhibited granulosa cell proliferation. Furthermore, the increase in PCNA protein and decrease in p27 protein were also observed in hLGCs of women with PCOS but not in those from control women.

Previous studies reported hyperandrogenemia could impede the proliferation of granulosa cells in PCOS animal models, which may induce the aberrant folliculogenesis. The result of our study was in consistent with that. As an intergenic IncRNA, LINC-01572:28 is highly expressed in testis and positive related with the concentration of testosterone in serum of women with PCOS. Thus, we think LINC01572:28 might be a mediator or treatment target in this pathway.

Implications of all the available evidence

In the current study, our findings showed that LINC-01572:28 was higher in granulosa cells of women with PCOS, and that its up-regulation was associated with hyperandrogenemia. LINC-01572:28 may have suppressed the proliferation of granulosa cells partly by decreasing the degradation of the p27 protein. Our study not only demonstrated the role of LINC01572:28 involved in the etiology of PCOS, but also provided a novel interaction mode for IncRNAs, meanwhile, a potential target for the treatment of PCOS.

2. Materials and methods

2.1. Subjects

Ovarian granulosa cells were collected from patients who underwent in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) at the Center for Reproductive Medicine of Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine. The study was approved by the ART Ethics committee of Ren Ji hospital, school of medicine, shanghai jiao Tong university (number: 2017041411), and informed consent was obtained from all participants. Anthropometric variables, such as age, body mass index (BMI), and select endocrine and biochemical parameters were recorded and are presented in Table 1. A diagnosis of PCOS was based on the revised Rotterdam diagnostic criteria for PCOS [35][35]. All patients in the non-PCOS control group had regular menstrual cycles (26–35 days) and normal ovarian morphology by ultrasound examination. Follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone (T), estradiol (E2), and anti-Müllerian hormone (AMH) levels were determined using either chemiluminescent assay (Beckman Access Health Company, Chaska, MN) or ELISA (Kangrun, Guangzhou, China) kits.

2.2. Collection of follicular fluid and culture of primary ovarian granulosa, KGN, and SVOG cells

Ovarian stimulation and oocyte retrieval were performed under a gonadotropin-releasing hormone (GnRH) antagonist or agonist protocol. After adequate follicle development, as detected by both ovarian ultrasound and serum estradiol assay, human chorionic gonadotropin (Lyzhu, Zhihui, China) was administered to trigger ovulation. Ultrasound-guided oocyte retrieval was performed 36 h later. Human luteinized granulosa cells (hLGCs) were retrieved from the follicular fluid as previously described [43]. The follicular fluid was pooled and centrifuged at 2500 rpm for 15 min, then the pellets were re-suspended in phosphate-buffered saline (PBS) and dispersed in 0.1% hyaluronidase (Sigma Chemical Co., St. Louis, MO, USA) at 37 °C for 15 min. Granulosa cells then were purified by Ficoll-Paque (GE
2.3. Microarray analysis

The hLGCs from three women with PCOS and three BMI, age, and treatment-matched reproductive normal, control women were selected for microarray analysis (Affymetrix HTA 2.0 Array). The microarray contained >6,000,000 probes covering 44,699 coding genes and 22,829 non-coding genes. Both the design and annotation of the probes were derived from authoritative databases, including RefSeq, Ensembl, UCSC (known genes and lincRNA transcripts), Mammalian Gene Collection (MC v10), NONCODE, IncRNA db, the Broad Institute Human Body Map lincRNAs, and the TUCP catalog (Broad Institute). The microarray analyses were performed by Biotechnology Corporation (Shanghai, China) as described in the supplementary materials. Significant differentially expressed transcripts were filtered by both p-value and fold change (\(p < .05\) and fold change \(>1.5\), respectively) between PCOS and control samples.

2.4. Transfection of cells

KGN cells, SVOG cells, and hLGCs (\(2 \times 10^5\)) were seeded into six-well plates and cultured for 1 day and then transfected with siRNAs and/or plasmid DNA using Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s protocol. After transfection, the cells were incubated for 48 or 72 h before further treatment.

2.5. RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was extracted from cells by TRIzol (Invitrogen) and then reverse transcribed into cDNA (PrimeScript™ RT reagent Kit, Takara, Dalian, China). Target gene expression was detected by quantitative real-time PCR for LINC01572:28. Cohort 4 represents the GCs which are used for clinical characters analysis. Data are presented as mean±SD, BMI: Body mass index, LH: luteinizing hormone, FSH: follicle stimulating hormone, T: testosterone. 

2.6. Western blot assay

Cells were harvested and lysed in ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (Shenggong, Shanghai, China) containing a protease inhibitor cocktail (Shenggong) and phosphatase inhibitor (Shenggong). The protein was extracted from cells and quantified as previously described [43] using 10−15% SDS-polyacrylamide gel. Relative band density was detected by Gel-Pro Analyzer (Media Cybernetics, L.P.) and normalized to β-actin Ab (Proteintech, Wuhan Hubei, P.R.C). Especially, the relative phosphorylation of target protein was calculated by the ratios of phosphorylated protein to target protein. The antibodies used in the Western blot analysis are summarized in Supplementary Table S2.

2.7. Cell proliferation assay

After transfection for 24 h, cells were harvested by trypsinization. The CCK-8 assay was used according to the manufacturer’s protocol to determine cell viability. The absorbance of the solution was measured at 450 nm. Ethynyl-2-deoxyuridine (EdU) assays were performed using an EdU Cell Proliferation Assay Kit (RiboBio, Guangzhou, China) according to the manual book. Cell proliferation was analyzed under a fluorescent microscope (Zeiss, Jena, Germany).

2.8. Flow cytometry analysis

For cell cycle analysis, KGN cells were harvested by trypsinization 48 h after transfection with siRNAs and/or plasmid DNA, then centrifuged and suspended in 1 mL ice-cold PBS. The cells were washed twice by PBS, and then the cells were re-suspended using 70% ice-cold ethanol and kept at 4 °C overnight. The fixed cells were centrifuged and the supernatant fluid was discarded. The cell sediment then was re-suspended in 500 µL propidium iodide with RNase A. After incubation at room temperature for 15 min, the cells were filtered once through 400 mesh sieves and then subjected to flow cytometry analysis. The results were analyzed by ModFIT LT 2.0.

2.9. RNA immunoprecipitation

RNA immunoprecipitation (RIP) experiments were performed using a Magna Nuclear RIP™ (Cross-Linked) Nuclear RNA-Binding Protein Immunoprecipitation Kit (Millipore, Burlington, MA, USA) according to the manufacturer’s instructions. EZH2 and SKP2 antibodies (Proteintech, Wuhan Hubei, P.R.C) were used for the immunoprecipitation.
Fig. 1. LINC01572:28 is upregulated in granulosa cells of the PCOS patients. (A) Heat-map showing the hierarchical clustering of differentially expressed genes in patients with and without PCOS: red: upregulated genes; green: downregulated genes. (B) Graph showing the biotypes of non-coding RNAs profiled. (C) The differential expression of lncRNAs from microarrays and qRT-PCR respectively. (D) Graph showing the levels of the selected lncRNAs in 10 PCOS patients and 10 healthy controls. The expression levels were detected via qRT-PCR and normalized against β-actin. (E) Graph showing the correlation between the expression level of LINC01572:28 in hLGCs and the serum concentration of testosterone in patients with PCOS. The expression level was detected via qRT-PCR. The first ΔCt normalization was to β-actin, and the 2nd ΔCt normalization was to the control group in cohort 3. Error bars represent SDs of at least 3 independent experiments. ***: p < .001, **: p < .01, *: p < .05, and ns (not significant; P > .05) correspond to two-tailed Student’s tests.
2.10. Co-immunoprecipitation

Co-immunoprecipitation (co-IP) was performed in lysates prepared from transfected cells (100 μg total protein) using either the SKP2 antibody or normal rabbit IgG at 4 °C overnight. On the next morning, the protein-antibody complex was incubated with 15 μL magnetic protein A + G beads for 1 h at 4 °C with gentle rotation. The antibody-protein-bead complexes then were washed three times with co-IP buffer. The protein in the complex then was eluted with 20 μL 1× loading buffer and boiled before running on a 15% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose membranes, and SKP2-associated p27 protein was immunoblotted using antibodies against p27.

2.11. Statistical analysis

All calculations were performed using the SPSS statistical software package (version 19.0) (IBM Corp., Armonk, NY, USA). The data are presented as the mean ± standard deviation (SD). The Kolmogorov-Smirnov test was used to assess whether the data were of normal distribution. Statistical comparisons between women with and without PCOS were performed using two-tailed Student’s t-test. The Spearman’s correlation and linear regression analyses were used to examine the association between gene expression levels and the distributions of relevant variables. Statistical comparisons between two groups of cells (the experimental group and the control group) were performed using the Student’s t-test. P < .05 was considered statistically significant. Statistical significance was evaluated using data from at least three independent experiments.

3. Results

3.1. Evaluation of clinical characteristics

A total of 132 subjects (89 women with PCOS and 43 normal women) were included in this study (3 PCOS patients and 3 controls...
Fig. 3. LINCO1572–28 induced cell cycle arrest at G0-G1 phase in granulosa cells. (A) The granulosa cells were transfected with p-LNC or p-Enter for 48 h; the effect on cell cycle was assessed by PI staining followed by Modfit analysis. (B) qRT-PCR analysis for CCNE1 and CCND1 mRNA level in p-LNC and p-Enter KGN and SVOG cells. The expression level was normalized against β-actin. (C) Western blot analysis using Cyclin E1 and Cyclin D1 antibodies on protein extracts from granulosa cells after transfected with vectors for 48 h. (D and E) The levels of phosphorylated CDK2 and Rb in p-LNC and p-Enter granulosa cells were assessed by immunoblotting. Error bars represent SDs from at least 3 independent experiments. ***: \( p < .001 \), **: \( p < .01 \), *: \( p < .05 \), and ns (not significant; \( P > .05 \)) correspond to two–tailed Student’s tests.
Fig. 4. Involvement of p27 in LINC01572:28-induced proliferation arrest of granulosa cells. (A and B) The mRNA and protein level of p27 and p21 in granulosa cells after transfection with vectors for 48 h. (C) p-LNC and p-Enter cells were treated with si-Ctrl or si-p27 for 48 h; the proportion of proliferation cells was assessed by EdU assay in granulosa cells. (D) p-LNC and p-Enter cells were treated with si-Ctrl or si-p27 for 48 h; the effect on cell cycle was assessed by PI staining followed by Modfit analysis. Error bars represent SDs from at least 3 independent experiments. ***: p < .001, **: p < .01, *: p < .05, and ns (not significant; P > .05) correspond to two–tailed Student’s tests.
for microarray analysis and the others for qRT-PCR). The major anthropometric variables and endocrine parameters of the women are presented in Table 1. Women with PCOS had significantly higher serum levels of LH and testosterone compared with the control group. These findings are consistent with the typical features of PCOS.

3.2. Expression profiles of differentially expressed transcripts

To identify transcripts that are potentially involved in the etiology of PCOS, we evaluated whole-transcript expression profiles in hLGCs by microarray analysis. As shown in Fig. 1A, most of the non-coding transcripts in the expression profile were not annotated. A total of 128 non-coding transcripts and 22 protein-coding transcripts exhibited differential expression between patients with and without PCOS. Among the 128 non-coding transcripts, 77 were lncRNAs, 14 were piRNAs, 4 were snoRNAs, 4 were miRNAs, and 18 were tRNAs (18) (Fig. 1B).

To validate the microarray results, six differentially expressed lncRNAs were selected and analyzed in a cohort of 10 women with PCOS and 10 treatment-matched reproductively normal control women using qRT-PCR (Fig. 1C, D). The expression levels of LINC01572:28, lnc-EIF2B5–7-1, lnc-SIK2–1, lnc-CCNL1–3-1, and lnc-LYZL9-1 were increased in patients with PCOS (P < .05 for all lncRNAs), which was consistent with the microarray results. The top 20 differentially expressed lncRNAs and mRNAs are reported in Supplementary Table S2 and the microarray data have been uploaded to Gene Expression Omnibus database (accession number: GSE114419).
Fig. 6. LINC01572:28 stabilize p27 by interacting with SKP2 in granulosa cells. (A) qRT-PCR for LINC01572:28 from nuclear and cytoplasmic fractions of untreated granulosa cells. The cytoplasmic β-Actin and nuclear U6 were used as controls. (B) The enrichment of LINC01572:28 was measured by qRT-PCR from SKP2 RNA IPs (RIP) performed from formaldehyde-crosslinked granulosa cells. Immunoglobulin G (IgG) IP was used as negative controls and EZH2 IP was used as positive controls. (C) SKP2-p27 interaction was examined in KGN and SVOG cells by immunoprecipitation SKP2 from whole-cell lysates followed by immunoblotting for p27 and SKP2. About 5% of cell lysates used for co-IP were loaded as the inputs. IgG IP was used as control. Error bars represent SDs from at least 3 independent experiments. ***: p < .001, **: p < .01, *: p < .05, and ns (not significant; P > .05) correspond to two-tailed Student’s tests.
3.3. Elevation of novel lncRNA LINC-01572:28 in hLGCs from women with PCOS

LINC-01572:28 was one of the up-regulated lncRNAs identified from the hLGCs of patients with PCOS. To further validate the increase in LINC-01572:28, we investigated the expression level of LINC-01572:28 in another cohort of 30 women with PCOS and 30 controls (Fig. 1E). We also analyzed the correlation between clinical characteristics of PCOS and the expression level of LINC-01572:28 in a separate group of patients with PCOS (46 samples) (Fig. 1F). As shown in Fig. 1F, LINC-01572:28 was significantly positively correlated with testosterone concentration in women with PCOS (Spearman’s rho = 0.459, P = .001). No significant correlations between LINC-01572:28 and other clinical characteristics were observed.

3.4. Overexpression of LINC-01572:28 suppressed granulosa cell proliferation and cell-cycle progression in vitro.

To investigate the role of LINC-01572:28 in the development of PCOS, we first identified whether LINC-01572:28 is a potential coding gene. The PhyloCSF score for LINC-01572:28 was −154.2917, indicating that LINC-01572:28 is 10^{15.2917} times more likely to be a non-coding gene.
sequence than a coding sequence [19]. Consistently, evaluation with the Coding-Potential Assessment Tool identified a 2.53% probability that LINC-01572:28 is a coding sequence [40].

To evaluate the effects of LINC-01572:28 on cell biological function, we measured cell proliferation after transfection of the LINC-01572:28 overexpression vector into KGN cells, SVOG cells, and hLGCs. A blank loaded vehicle vector (p-Enter) was used as the control. The efficiency of the vector transfection was confirmed by qRT-PCR (Fig. 2A). We found that the abundance of PCNA protein was reduced in the overexpressed LINC-01572:28 granulosa cells (Fig. 2B). Edu and CCK-8 assays consistently showed that over-expressed LINC-01572:28 decreased the proportion of proliferating cells in total cells (Fig. 2C) and inhibited cell proliferation ability (Fig. 2D). We also examined whether the cell cycle was affected by LINC-01572:28 with propidium iodide staining via flow cytometry analysis and found that overexpression of LINC-01572:28 significantly induced G0-G1 phase arrest and decreased the percentage of cells in the S phase (Fig. 3A). Western blot analyses consistently showed that overexpression of LINC-01572:28 had no effect on the expression of cyclin D1, a factor involved in G0/G1 transition [24,41], but decreased the abundance of cyclin E1, a factor involved in G1/S transition [39], in KGN and SVOG cells (Fig. 3B and C). Furthermore, the phosphorylation of CDK2 and Rb, two factors involved in cell cycle progression, also were reduced upon overexpression of LINC-01572:28 in KGN and SVOG cells (Fig. 3D and E). These data suggest that LINC-01572:28 may inhibit cell proliferation via G0-G1 phase arrest in granulosa cells.

3.5. Involvement of p27 in LINC-01572:28-induced proliferation arrest of granulosa cells

Cyclin-dependent kinase inhibitor 1A (p21) and 1B (p27) are two factors related to the G1/S transition that may inhibit the activity of the cyclin E/CDK2 complex. We examined whether the overexpression of LINC-01572:28 affects the expression of these two factors. LINC-01572:28 had no effect on p21 (CDKN1A) mRNA and protein or p27 (CDKN1B) mRNA expression (Fig. 4A), but significantly induced an abundance of p27 protein in KGN and SVOG cells (Fig. 4B).

Next, we conducted rescue assays to determine whether p27 is involved in the LINC-01572:28-induced cell proliferation arrest. siRNA-mediated knockdown of p27 expression significantly increased the proliferation rate (Fig. 4C), reduced the G0-G1 phase arrest (Fig. 4D), increased the abundance of PCNA and cyclin E1, and increased the phosphorylation of CDK2 and Rb (Fig. 5) in KGN and SVOG cells. The efficiency of the p27 knockdown is shown in Supplementary Fig. S1. Furthermore, the effects of the overexpression of LINC-01572:28 on the cell proliferation rate (Fig. 4C), G0-G1 phase arrest (Fig. 4D), PCNA and cyclin E1 abundance, and CDK2 and Rb phosphorylation were rescued by the siRNA-mediated knockdown of p27 in KGN and SVOG cells (Fig. 5). These results suggest that LINC-01572:28 inhibits granulosa cell proliferation at least in part due to an increase in p27 protein abundance.

3.6. LINC-01572:28 stabilized p27 by interacting with SKP2 in granulosa cells

To determine the molecular mechanism underlying the regulation of p27 protein by LINC-01572:28, we first detected the subcellular location of LINC-01572:28 in granulosa cells. The cytoplasmic and nuclear RNA were separated and reverse transcribed, which revealed that LINC-01572:28 was located predominantly in the nucleus in both KGN and SVOG cells (Fig. 6A). ACTB and U6 were used as cytoplasm and nucleus controls, respectively. Because overexpression of LINC-01572:28 did not affect p27 mRNA expression, we hypothesized that LINC-01572:28 may inhibit the degradation of the p27 protein. Previous studies have demonstrated that SKP2, a key component of the SCFSKP2 ubiquitin-ligase complex, may mediate the cyclin E/CDK2 dependent ubiquitination and degradation of p27 by binding to p27 [23,30,37]. LINC-01572:28 was shown to possess the ability to bind to SKP2 using the online algorithm RPISeq. Thus, we hypothesized that LINC-01572:28 may stabilize p27 by interacting with SKP2 in granulosa cells. Khail et al. reported that approximately 20% of IncRNAs bind to PRC2, which includes EZH2. We therefore used the EZH2-HOTAIR interaction pair as a positive control (interaction probability = .75) [26]. Using RPISeq, we found that the LINC-01572:28-SKP2 interaction pair had a higher score (interaction probability = .9) than LINC-01572:28-EZH2 (interaction probability = .75). Furthermore, RIP results showed a more significant enrichment of LINC-01572:28 using the SKP2 antibody compared with both the EZH2 antibody and the non-specific IgG control antibody (Fig. 6B) in both KGN and SVOG cells. In addition, the co-IP results demonstrated that the overexpression of LINC-01572:28 reduced the interaction of SKP2 with p27 in both KGN and SVOG cells (Fig. 6C). These data suggest that LINC-01572:28 may increase p27 protein abundance by reducing the degradation of p27 protein via an interaction with SKP2 in granulosa cells.

3.7. Decrease in PCNA expression and increase in p27 expression in granulosa cells in patients with PCOS

We further tested the PCNA and p27 expression in the luteinized granulosa cells of patients with and without PCOS. As shown in Fig. 7A, the PCNA protein level was decreased whereas the p27 protein was increased in the granulosa cells obtained from women with PCOS.

4. Discussion

In the current study, we demonstrated that the abundance of LINC-01572:28 was elevated in the hLGCs of women with PCOS compared to those from control women using microarray and qRT-PCR. The elevation of LINC-01572:28 impeded the interaction between SKP2 and p27 via competitive binding to SKP2, which resulted in the accumulation of p27. The accumulation of p27 in turn induced cell cycle arrest and inhibited granulosa cell proliferation. Furthermore, the decrease in PCNA protein and increase in p27 protein were also observed in hLGCs of women with PCOS but not in those from control women.

The arrest of follicle growth is considered the main cause of chronic oligo/anovulation in women with PCOS who exhibit disordered folliculogenesis [4,18,29]. Granulosa cells, which play an important role in folliculogenesis by providing nutrients and growth factors to developing oocytes, are regulated by FSH during the antral follicle phase [5,17,28]. Previous studies have suggested that FSH stimulates the proliferation of granulosa cells via the up-regulation of PCNA protein [42]. In addition, PCNA is down-regulated in the granulosa cell layers of rat and bovine PCOS [2,3,13,36], potentially impairing granulosa cell proliferation. Consistent with these animal models of PCOS, in the present study, we detected a decrease in PCNA protein levels in the luteinized granulosa cells of women with PCOS.

p27 is a cyclin-dependent kinase inhibitor, which negatively regulates G1 phase progression. Previous studies have reported that p27 may act as a tumor suppressor by reducing the phosphorylation of Rb (a negative regulator of the cell cycle which can be inactivated after phosphorylation) via binding to cyclin E/CDK2 or cyclin D/CDK4 complexes [16,38]. Previous studies also have shown that FSH may induce granulosa cell proliferation by regulating p27 expression via the PI3K/Akt and MAPK/ERK pathways [12,14]. p27 also was shown to negatively regulate the activation of murine primordial oocytes in ovarian granulosa cells [8]. Moreover, Robker et al. reported that LH could terminate follicular growth via the up-regulation of p27 and p21 in rhesus monkeys [34]. In the present study, we demonstrated that the siRNA-mediated knockdown of p27 consistently induced granulosa cell proliferation, suggesting that p27 plays a critical role in granulosa cell proliferation. In addition, p27 was more abundant in women with PCOS,
suggesting that p27 appears to be involved in the arrest of follicle progression characteristic of PCOS.

In this study, we found that LINC-01572:28 could regulate p27 protein level due to its high affinity to SKP2, a key component of the SCF<sup>SKP2</sup> ubiquitin-ligase complex that mediates the cyclin E-CDK2 dependent ubiquitination and degradation of p27 [23,30,37]. Previous studies of proliferation-related IncRNAs, which were performed mainly in tumor cells, showed that IncRNAs could function via an interaction with RNA-binding proteins to influence the transcriptional activity of the target gene, either epigenetically or through the direct stabilization of the mRNA or protein [9,31]. In the present study, we found that IncRNA also could regulate the target gene via competitive binding to a key component of the ubiquitination complex, resulting in a decrease in the degradation of the target protein. This finding thus identifies a new interaction mode for IncRNAs providing additional insight into their function.

LINC01572:28 is an intragenic IncRNA, which is highly expressed in testis [15]. Meanwhile, hyperandrogenemia is not only a key character of PCOS, but also a leading cause of subfertility or infertility. Furthermore, we found that the abundance of LINC01572:28 in the granulosa cells of women with PCOS are positive related with the concentration of testosterone in serum. All of these clues indicated that LINC01572:28 might play a role in the etiology of PCOS, thus, we chose this IncRNA as the studying object. However, we could not elucidate the relationship between the LINC01572:2 and hyperandrogenemia due to the unavailability of sufficient human theca cell, the main source of androgen in ovary. What’s more, it seems that overexpressed LINC01572:28 did not influence the abundance of CYP19A1 protein (Supplementary Fig.6), an enzyme responsible for a key step in the biosynthesis of estrogens, is responsible for the aromatization of androgens into estrogens. So, the relationship between the elevation of LINC01572:28 in granulosa cells of women with PCOS and androgen excess was still vague and needed to be elucidated in the future study.

The relationship between hyperandrogenemia and granulosa cell proliferation arrest had been reported several times [2,3], and LINC01572:28 might be a mediator or treatment target in this pathway. As FSH could stimulate granulosa cells to grow, we also detected the abundance of FSHR and LHCR mRNA in the LINC01572:28 overexpressed granulosa cells, and it showed that there was no difference between these two groups (Supplementary Fig.5). Besides the dysfunction of p27/CDK2 and Cyclin E/Rb axis in the LINC01572:28 overexpressed cell, we also found that mRNA level of Cyclin D1 is downregulated, but there is no change of Cyclin D1 protein level. These indicate LINC01572:28 may influence the degradation of Cyclin D1 protein at the same time, so we could not observe a consistent change in mRNA and protein level of Cyclin D1 after overexpression of LINC01572:28 in KGN cells. Of note, Liu et al. detected the transcripts in a cohort which includes 7 PCOS patients and 7 controls and found that a total of 862 IncRNA were differentially expressed [20], however, we only identified 77 differentially expressed IncRNA between control and PCOS patients in this current study, we think the difference may come from the sample size, the clinical characters and treatment protocol of the PCOS patients, and the different microarray platforms from two companies.

In summary, our findings showed that LINC-01572:28 was higher in granulosa cells of women with PCOS, and that its up-regulation was associated with hyperandrogenemia. LINC-01572:28 may have suppressed the proliferation of granulosa cells partly by decreasing the degradation of the p27 protein. Our study provides a new perspective regarding the molecular mechanisms underlying the disordered folliculogenesis characteristic of PCOS and identifies LINC-01572:28 as a novel target for the treatment of PCOS.

**Authors’ contributions**

Yan Zhi Du and Zi-jiang Chen conceived and supervised the study. Jieying Xu assisted with microarray analysis. Jun Zhao, Wangsheng Wang, Xiaojing Liu and Jiansheng Liu designed and conducted the study, including acquisition, analysis, and interpretation of data. Han Zhao, Yun Sun and Hongbui Liu participated in the sample collecting and kindly provided cell lines. Jun Zhao and Wangsheng Wang drafted the manuscript. Andrea Dunai and Yan Zhi Du commented on and revised the drafts of the manuscript. All authors critically reviewed, edited and approved the manuscript.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.09.043.

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