Lnc-GULP1-2:1 affects granulosa cell proliferation by regulating COL3A1 expression and localization

Guidong Yao (ygdzzu@163.com)
The First Affiliated Hospital of Zhengzhou University https://orcid.org/0000-0002-7174-9989
Yue Kong
Zhengzhou University First Affiliated Hospital
Guang Yang
Zhengzhou University First Affiliated Hospital
Deqi Kong
Zhengzhou University First Affiliated Hospital
Yijiang Xu
Zhengzhou University First Affiliated Hospital
Jiahuan He
Zhengzhou University First Affiliated Hospital
Ziwen Xu
Zhengzhou University First Affiliated Hospital
Yucheng Bai
Zhengzhou University First Affiliated Hospital
Huiying Fan
Zhengzhou University First Affiliated Hospital
Qina He
Zhengzhou University First Affiliated Hospital
Yingpu Sun
Zhengzhou University First Affiliated Hospital

Research

Keywords: lncRNA, COL3A1, ovarian follicular development, granulosa cell, cell proliferation

Posted Date: December 18th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-72763/v2

Version of Record: A version of this preprint was published on January 20th, 2021. See the published version at https://doi.org/10.1186/s13048-021-00769-1.

Abstract

Backgrounds: Long non-coding RNA is a kind of RNA molecule with a transcript length of more than 200 nt and lacking protein coding ability. Recent studies have found that it is widely involved in many pathological and physiological processes. In our previous study, we found that lnc-GULP1-2:1 was significantly down-regulated in the ovarian cortical tissue of patients with primary ovarian insufficiency and predicted that lnc-GULP1-2:1 has a regulatory effect on COL3A1.

Results: In this study, we found that lnc-GULP1-2:1 was mainly localized in the cytoplasm of luteinized granulosa cells and was lower expressed in patients with diminished ovarian reserve but highly expressed in patients with polycystic ovary syndrome. Overexpression of lnc-GULP1-2:1 in KGN cells significantly inhibited cell proliferation, which may be related to the regulation of cell cycle related genes CCND2 and p16. To further investigate the regulation of lnc-GULP1-2:1 on COL3A1, RNA analysis revealed a positive correlation between the expression of lnc-GULP1-2:1 and COL3A1 in multiple cell lines, and this was consistent in luteinized granulosa cells from patients with different ovarian functions. We also found that overexpression of lnc-GULP1-2:1 in KGN cells promoted the expression and migration of COL3A1 into the nucleus, and silencing COL3A1 gene in KGN cells also significantly inhibited cell proliferation.

Conclusions: Lnc-GULP1-2:1 affects the proliferation of granulosa cells by regulating the expression and localization of COL3A1 protein, and may participate in the regulation of ovarian follicle development. This study will provide a new idea for understanding the regulatory mechanism of follicular development and a new strategy for the diagnosis and treatment of diseases related to ovarian follicular development disorders in the future.

Introduction

Long non-coding RNA (lncRNA) is a kind of RNA molecule with a transcript length of more than 200 nt and lacking protein coding ability [1-3]. It should be noted that lncRNA is a generic term that encompasses different classes of RNA transcripts, including enhancer RNA, snoRNA hosts, intergenic transcripts, and sense or antisense transcripts that overlap other transcripts [4]. Unlike miRNAs or proteins, the function of most lncRNAs cannot yet be inferred from the sequence or structure [5]. At present, the main regulatory mechanisms of lncRNA are chromatin modification, transcriptional and post-transcriptional regulation [5, 6].

LncRNAs are involved in a variety of biological functions and pathological processes, including proliferation, differentiation, development, apoptosis and carcinogenesis [7-9]. For example, overexpression of lncRNA-Amhr2 in mouse granulosa cells resulted in decreased mRNA levels of Amhr2, and activation of lncRNA-Amhr2 increases Amhr2 promoter activity [8]. Furthermore, the expression of lncRNA and its regulation of downstream target genes have higher tissue and organ specificity. Most of the current studies are focused on the identification of new lncRNAs, but functional studies on specific lncRNAs are very limited, especially in human ovarian granulosa cells.

Primary ovarian insufficiency (POI) refers to the loss of function of the ovary before the age of 40, accompanied by amenorrhea, hypergonadotropism and hypoestrogenism [10, 11]. In most cases, the specific mechanism leading to premature depletion of the primordial follicle pool is unclear. Genetic disorders, autoimmune diseases, tuberculosis of the genital tract, smoking, ovarian surgery, radiation and/or chemotherapy are potential causes of POI [11]. Although many genetic mutations and toxic agents are destructive to oocytes, the relationship between oocyte destruction and POI and the role of these factors in ovarian function and POI remain to be explained.

During follicular development, granulosa cells surround the oocyte and differentiate into mural granulosa cells and cumulus granulosa cells [12]. The physiological function of granulosa cells depends on paracrine and autocrine cytokines in the ovarian microenvironment and reproductive hormones in peripheral blood [13, 14]. Oocytes and granulosa cells grow and develop in a highly coordinated, interdependent manner [15-18]. Granulosa cells provide nutrient and maturation-related factors to oocytes, ensuring oocyte development and maturation [19]. Apoptosis of granulosa cells is a physiological phenomenon in follicles. The decrease of the number of granulosa cells and the destruction of cell communication may cause oocytes in pre-ovulatory follicles lacking nutrients and survival factors, thus leading to oocyte apoptosis and follicular atresia [20], and eventually result in a decrease in the number of follicles [21]. The gradual decrease in the number of granulosa cells is a key factor in antral follicle atresia. Therefore, abnormality of ovarian granulosa cells is a key step leading to abnormal follicular development [22, 23].

In our previous study, next generation sequencing revealed that lnc-GULP1-2:1 and collagen type III alpha 1 chain (COL3A1) were significantly down-regulated in ovarian cortical tissue in patients with POI than normal ovarian cortical tissue [24]. Co-expression network analysis showed that COL3A1 was the *cis*-predicted transcript of lnc-GULP1-2:1 [4, 25]. The full length of lnc-GULP1-2:1 is 628bps (the detailed information of lnc-GULP1-2:1 is provided in supplementary material), which overlaps with the 5’ end of COL3A1 gene [24]. Among them, 1-378bps of lnc-GULP1-2:1 overlaps with exon 1 and 2 of COL3A1 gene, 379-612bps of lnc-GULP1-2:1 completely overlaps with exon 2 and the intron between exon 2 and 3 of COL3A1 gene, and 613-628bps of lnc-GULP1-2:1 partially overlaps with exon 3 of COL3A1 gene. Through sequence analysis and comparison, we believe that lnc-GULP1-2:1 may be the alternative splicing product of COL3A1 gene.

Therefore, we speculate that lnc-GULP1-2:1 may affect the function of granulosa cells by regulating COL3A1 expression. In view of this, this study explored the expression level of lnc-GULP1-2:1 in granulosa cells of patients with different ovarian functions and its influence on granulosa cell proliferation and cell cycle regulation, and further studied the potential mechanism of lnc-GULP1-2:1 by regulating COL3A1 to affect cell proliferation.

Materials And Methods

Sample collection

A total of 22 patients were enrolled, including 7 patients of the normal group, 6 patients of the diminished ovarian reserve (DOR) group and 9 patients of the polycystic ovary syndrome (PCOS) group. The patients in normal group were with normal menstrual cycles, younger than 35 years old; DOR group were patients affected by secondary infertility and older than 40 years [24]; and the included PCOS patients were younger than 35 years old and were diagnosed according to the Rotterdam 2003 criteria [26], which require a minimum of two of the following three criteria: ultrasound demonstration of polycystic ovaries, chronic anovulation and hyperandrogenism. All the enrolled patients underwent follicular aspiration for the first time and had no history of ovarian surgery.

Primary luteinized granulosa cell isolation and cell culture

Density gradient centrifugation (Lymphocyte Separation Medium, LTS1077, Tianjin, China) was used to remove the mixed red blood cells and lymphocytes in the follicular fluid obtained by follicular aspiration to obtain pure luteinized granulosa cells. Then cell sedimentation was washed twice with ice-cold phosphate-buffered saline (PBS), and resuspended in DMEM/F12 (Gibco, Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Gibco) and 50 U/mL penicillin-streptomycin (Gibco). KGN, a human granulosa-like tumor cell line, was gifted from Prof. Fei Sun (University of Nantong, China). Lentivirus KGN cell line stably overexpressing lnc-GULP1-2:1 (Lv-lnc-GULP1-2:1) and its control (Lv-EGFP) was constructed using the similar method as in previous studies [24]. KGN, MDA-MB-231 cells were cultured in DMEM/F12 medium (Gibco). 293T, BeWo and HTR-8/SVneo cell lines were cultured in high-glucose DMEM containing glutamax1 (Invitrogen, Paisley, UK). OVCAR3 cells were cultured in Low Glucose DMEM and SKOV3 cells were cultured in RPMI1640 containing glutamax1 (Invitrogen). U87 MG and Hep G2 cells were cultured in MEM (Gibco). JAR was cultured in RPMI-1640 medium (Invitrogen). All the cell lines used in this study have been verified by short tandem repeat (STR) analysis to verify its authenticity, and all cell lines were routinely cultured at 37℃ in an atmosphere of 5% CO_2 in compressed air at high humidity and all media were supplemented with 10% fetal bovine serum (FBS, Life Technologies) and 50 U/mL penicillin-streptomycin (Life Technologies).

Quantitative RT-PCR

Total RNA was extracted by using Trizol reagent (Life technologies, Inc., Gaithersburg, MD, USA). Total RNA (approximately 800 ng) was reverse transcribed into cDNA by using the iScript™ cDNA Synthesis Kit (Bio-Rad, CA, USA). Briefly, the 20 μl RT reactions (0.8 μg RNA, 5 μl iScript reaction Mix, 1 μl iScript Reverse Transcriptase and ddH_2O) was incubated for 5 min at 25℃ and 20 min at 46℃, incubated for 1 min at 95 ℃ and then maintained at 4℃. For real-time PCR, all reactions were performed in triplicate with iTaq™ Universal SYBR® Green Supermix (Bio-Rad) under the following conditions: 30s at 95℃ for initial denaturation, followed by 40 cycles of segments of 95 ℃ for 3s and 60 ° C for 30s in 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference gene. Gene expression was calculated by using the method of $2^{-\triangle\triangle Ct}$, $\triangle Ct = \triangle Ct_{target} - \triangle Ct_{reference}$, $-\triangle\triangle Ct = -(\triangle Ct_{sample} - \triangle Ct_{control})$. The sequences of primers for real-time PCR are listed in Supplementary Table 1.

Cell proliferation assays

The cells were inoculated at a density of 1×10^4 cells in 96-well plates, and cultured for 24 h, followed by corresponding treatment. Then, cell proliferation was measured by using Cell Counting Kit-8 (Boster, Wuhan, China) at 0, 24, 48 and 72 h after treatment. At the indicated time, fresh 100 μl DMEM/F12 plus 10 μl CCK-8 medium was added to each well, incubated for 2 h in cell incubator, and OD value was measured at 450 nm using spectrophotometer (Thermo Fisher, Vantaa, Finland). Each experiment was independently repeated 3 times, and each treatment had 6 replicate wells in each group.

Western blot

Total Protein Extraction Kit (Sangon Biotech, Shanghai, China) was used for total protein extraction according to production instructions. Protein concentrations were measured by using Dye Reagent (Bio-Rad) with Quick Start™ Bovine Serum Albumin (Bio-Rad) as standard. Then the samples were boiled in protein loading buffer (Boster) at 100℃ for 10 min, and equal amounts of protein (40 μg) was loaded into the wells of the SDS-PAGE gel. Following by electrophoresis according to standard procedures, the separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad) in a wet transfer system (Bio-Rad). The membrane was blocked for 1 h at room temperature in 5% non-fat dry milk in tris-buffered saline supplemented with 0.1% Tween-20 (TBST). After blocking, the membrane was incubated with primary antibody (anti-COL3A1 mouse monoclonal antibody, 1:100, Santa Cruz, Oregon, USA; anti-GAPDH mouse monoclonal antibody, 1:3000, CMCTAG, Milwaukee, USA) in blocking buffer at 4℃ overnight. Then the PVDF membranes were washed 3 times (10 min each) with TBST at room temperature and incubated with secondary antibody (goat polyclonal secondary antibody to mouse IgG-H&L (HRP), 1:5000, Abcam, Cambridge, USA) for 1 h. Finally, immunoreactive bands were detected by using enhanced chemiluminescent substrate in a ChemiDoc MP imaging system (Bio-Rad).

Immunofluorescence

Lentivirus-stabilized KGN cell lines, which stably overexpressing lnc-GULP1-2:1 (Lv-lnc-GULP1-2:1) and its control (Lv-EGFP), were re-inoculated on glass coverslips pre-coated with poly-lysine and cultured for 24 h in an atmosphere of 5% CO_2 at high humidity. Cells were treated with 4% paraformaldehyde for 20 min, then gently washed with phosphate buffered saline (PBS) for 3×3 min. Next, cells were permeabilized with 0.5% Triton X-100 in PBS for 20 min and blocked by using 5% bovine serum albumin (BSA) in TBST for 30 min at room temperature. After blocking, cells were incubated with COL3A1 antibody (1:100, Santa Cruz) or Ki-67 antibody (1:1000, Cell signaling technology, Beverly, USA) at 4℃ overnight and PBS was used as a negative control. Then cells were washed 3 × 3 min with PBS at room temperature and incubated with Alexa Fluor 594-AffiniPure Goat Anti-Rabbit IgG (1:600, Jackson, Pennsylvania, USA) for 30 min. After washing with PBS, the nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) (5 μg/ml). Then the coverslips were washed again with PBS 4 × 5 min. Finally, the coverslips were mounted

with anti-fade Mounting Medium (Beyotime Biotechnology, Shanghai, China), and the image was observed and collected under a laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

Fluorescence *in situ* hybridization

Cells were re-plated on poly-lysine-pre-coated glass coverslips and cultured for 24 h in an atmosphere of 5% CO_2 at high humidity, then treated with 4% paraformaldehyde for 20 min. After washing the coverslips, cells were digested by 20μg/ml proteinase K (Servicebio, Wuhan, China) for 5 min. Then the coverslips were washed 3 × 3 min with PBS at room temperature and incubated with prehybridization solution for 1 h at 37℃. Next, the prehybridization solution was discarded, and a hybridization solution (Servicebio) containing lnc-GULP1-2:1 probe (5'-DIG-CATGG CTATTTGATGAACATGACTTT-DIG-3') or nonsense control probe (5'-DIG GTGTAACACGTCTATACGCCCA-3') (Genepharma, Shanghai, China) at a concentration of 8 ng/μl, was added dropwise, and hybridization was carried out at 37℃ overnight. Then the hybridization solution was washed away and the coverslips was placed in 5% BSA in PBS (Servicebio) for 30 min. The blocking solution was discarded, anti-DIG-cy3 (Jackson, Pennsylvania, USA) was added dropwise, incubated at 37℃ for 50 min, and then washed 3 × 5 min with PBS. The DAPI staining solution was added to the coverslips, incubated for 8 min in the dark, and the anti-fade Mounting Medium (Servicebio) was added after washing. Finally, the slides were observed under a fluorescence microscope (NIKON ECLIPSE CI, Japan) and images were collected.

RNA interference

For gene knockdown analysis, COL3A1 specific siRNA (sc-43062, Santa Cruz) was used to silence the COL3A1 gene, and siRNA control (Silencer™ Select Negative Control, 4390843, Invitrogen) was used as a silence control. Cells were transfected with COL3A1 siRNA or control coupled with Lipofectamine® RNAi-MAX (Invitrogen) according the product manufacture protocol, and the cells were treated or collected at the indicated time for subsequent analysis.

Cell cycle analysis

Primary granulosa cells treated with 5×10^{10} PFU/ml adv-control or adv-lnc-GULP1-2:1 (Hanbio Biotechnology, Shanghai, China) for 48h were seeded (70% confluent) in 12-well plates for flow cytometry analysis. Cells were digested with EDTA-free trypsin, washed twice with cold PBS (1000 rpm × 5 min) and then fixed with 70% ethanol at 4 ℃ overnight. The fixed cells were centrifuged (1000 rpm × 5 min) again on the next day, resuspended in 500μl of PI/RNase solution（KeyGen Biotech，Nanjing，China), and then incubated in the dark for 30 minutes at 37 ℃. Flow cytometry studies were performed by using BD Accuri C6 Plus flow cytometer (BD Biosciences, San Jose, CA). The percentage of cells in different phases of cell cycle were analyzed by using FlowJo 10.4 (BD Biosciences).

Statistical analysis

Data were expressed as mean ± SD. All statistical analyses were performed using SPSS 22.0 (IBM, Armonk, NY, USA). Unpaired T-test with Welch's correction was used for the statistical analysis between the two groups, while one-way ANOVA followed by Bonferroni's post-hoc test was used for the statistical analysis of more than two groups of data. The difference of $p < 0.05$ was considered significant. Each experiment was repeated at least three times independently.

Results

The expression of lnc-GULP1-2:1 in luteinized granulosa cells is correlated with ovarian function status

In this study, we focused on the function of lnc-GULP1-2:1 and its regulation in ovarian follicular development. Luteinized granulosa cells were obtained from follicular fluid of IVF/ICSI patients isolated by using lymphocyte separation fluid, and we found that lnc-GULP1-2:1 is mainly localized in the cytoplasm of luteinized granulosa cells by using fluorescence *in situ* hybridization. However, lnc-GULP1-2:1 punctate expression signals can also be seen in the nucleus of some cells (Figure 1A).

Further, we collected luteinized granulosa cells from DOR and PCOS patients to explore whether the expression of lnc-GULP1-2:1 in luteinized granulosa cells is related to different ovarian function status. The results showed that the expression of lnc-GULP1-2:1 in luteinized granulosa cells of DOR patients was significantly lower than that of luteinized granulosa cells from normal patients ($p < 0.05$) (Figure 1B), while the expression of lnc-GULP1-2:1 in luteinized granulosa cells of PCOS was significantly higher than that of luteinized granulosa cells from normal patients ($p < 0.05$) (Figure 1C).

KGN cell proliferation can be regulated by overexpression of lnc-GULP1-2:1

To further evaluate the functional role of lnc-GULP1-2:1 in granulosa cells, lentivirus-stabilized KGN cell line capable of overexpressing lnc-GULP1-2:1 (Lv-lnc-GULP1-2:1) and the control group only capable of overexpressing EGFP (Lv-EGFP) were constructed. Results of real-time PCR showed that the KGN cell line with stable overexpression of lnc-GULP1-2:1 was successfully constructed (Figure 2A). By using cell counting kit-8 (CCK-8) to analyze the effect of Lv-lnc-GULP1-2:1 expression on KGN cell proliferation, we found that granulosa cell proliferation was significantly inhibited in lnc-GULP1-2:1 overexpressed KGN cells (Figure 2B). The immunofluorescence staining results of Ki-67 also showed that lnc-GULP1-2:1 inhibited the proliferation of KGN cells (Figure 2C). Genes related to cell proliferation, apoptosis and cell cycle were also analyzed, and the results showed that overexpression of lnc-GULP1-2:1 in KGN cells had no significant effect on the expression levels of apoptosis-inhibiting gene Bcl-2 and anti-apoptotic gene Bcl-XL, and the expression level of proapoptotic gene Bax ($p > 0.05$). However, the expression of the cell cycle-related genes was changed significantly, with the expression of cyclin D2 (CCND2) was significantly down-regulated, while p16 gene expression was significantly up-regulated (Figure 2D).

The expression and localization of COL3A1 can be regulated by lnc-GULP1-2:1 in granulosa cells

In order to further verify the regulatory relationship between lnc-GULP1-2:1 and COL3A1, we first investigated the expression correlation of these two genes in different human cell lines and found that there was a significant positive correlation between the expression of lnc-GULP1-2:1 and COL3A1 (R^2 = 0.7534, $p < 0.05$) (Figure 3A). In order to find out whether there is any difference in the expression of COL3A1 in luteinized granulosa cells from different patients, we analyzed the expression of COL3A1 in luteinized granulosa cells from normal, DOR and PCOS patients. The results showed that the expression of COL3A1 in DOR patient-derived luteinized granulosa cells was lower than that in the normal group but with no significant differences ($p > 0.05$) (Figure 3B). However, the expression of COL3A1 was significantly increased in PCOS patient-derived luteinized granulosa cells ($p < 0.05$) (Figure 3C).

Consistent with our previous results, lnc-GULP1-2:1 overexpression significantly upregulated both mRNA and protein levels of COL3A1 (Figure 3D & E). By immunofluorescence assay, we found that in Lv-EGFP control group, COL3A1 protein was mainly expressed in the cytoplasm of granulosa cells; while in Lv-lnc-GULP1-2:1 overexpression group, the protein expression level of COL3A1 was increased, which was consistent with the previous western blot results. More importantly, the intracellular localization of COL3A1 also changed significantly, and the expression of COL3A1 in the nucleus was significantly enhanced (Figure 3F).

Since lnc-GULP1-2:1 and COL3A1 have a certain sequence overlap, and lnc-GULP1-2:1 is involved in the regulation of COL3A1 expression. Therefore, further research is to explore whether the expression change of COL3A1 will affect the expression change of lnc-GULP1-2:1. By using COL3A1 specific siRNA to knockdown both the mRNA and protein expression of COL3A1 in KGN cells (Figure 3G & H), we found there is no significant difference in the expression of lnc-GULP1-2:1, suggesting that lnc-GULP1-2:1 expression is not regulated by COL3A1 (Figure 3I).

KGN cell proliferation is affected by COL3A1 protein expression level and intracellular localization

According to the abovementioned results, lnc-GULP1-2:1 may inhibit the proliferation of KGN cells by promoting protein expression of COL3A1. By knocking down the expression of COL3A1 gene in KGN cells, cell proliferation was not increased, but inhibited significantly in a dose-dependent manner (Figure 4A). Furthermore, simultaneous down-regulation of COL3A1 levels in Lv-lnc-GULP1-2:1 and Lv-EGFP cells further inhibited cell proliferation (Figure 4B). By analyzing genes related to cell proliferation, apoptosis and cell cycle, we found that down-regulation of COL3A1 did not affect the expression of Bcl-2, Bcl-XL and Bax genes; the expression of CCND2 gene was significantly inhibited by down-regulation of COL3A1 gene, and further reduced in Lv-lnc-GULP1-2:1 cell. Although p16 gene was significantly increased in Lv-lnc-GULP1-2:1 cell, the down-regulation of COL3A1 did not affect the expression level of p16 gene (Figure 4C). In addition, we tested the effect of lnc-GULP1-2:1 on cell cycle of primary granulosa cells by infecting cells with adenovirus overexpressing lnc-GULP1-2:1 (adv-lnc-GULP1-2:1) or its control (adv-control). The results showed that compared with the control group, the overexpression of lnc-GULP1-2:1 in granulosa cells increased the ratio of cells in G0/G1 phase and decreased the ratio of cells in G2/M phase (Figure

4D). These results suggest that lnc-GULP1-2:1 mainly causes the increased localization of COL3A1 in the nucleus, thereby inhibiting granulosa cell proliferation.

Discussion

In this study, we found that lnc-GULP1-2:1 expressed low in luteinized granulosa cells of patients with DOR, and *in situ* hybridization revealed that lnc-GULP1-2:1 mainly localized in the cytoplasm of luteinized granulosa cell and has a small amount of localization in the nucleus. By overexpressing lnc-GULP1-2:1 in KGN cells, we found that granulosa cell proliferation was significantly inhibited.

Both CCND2 and cyclin-dependent kinase inhibitor p16 are genes that play an important regulator role in the progression of the cell cycle. CCND2 interacts with the regulatory subunits of cyclin dependent kinase 4 (CDK4) or cyclin dependent kinase 6 (CDK6), and its activity is required for the G1 to S phase transition of the cell cycle [27], and p16 affects the cell cycle progress by slowing the transition from G1 to S phase [28]. By upregulation of lnc-GULP1-2:1 in granulosa cells, the expression of cell cycle-associated gene CCND2 was significantly decreased, while p16 expression was significantly up-regulated. Furthermore, flow cytometry analysis showed that overexpression of lnc-GULP1-2:1 increased the ratio of cell cycle in G0/G1 phase. These results suggesting that lnc-GULP1-2:1 inhibits cell cycle progression from G1 to S phase, thereby inhibiting cell proliferation.

Lnc-GULP1-2:1 is located at the 5' end of COL3A1 gene and overlaps partly with its transcripts, and our results showed that lnc-GULP1-2:1 affects the gene expression and protein localization of COL3A1. COL3A1 gene encodes the pro-alpha1 chains of type III collagen, a fibrillar collagen that is found in extensible connective tissues, which is an important component of the extracellular matrix [29]. Studies have shown that several miRNAs can play a role in different diseases by down-regulating the expression of COL3A1 and inhibiting the proliferation of corresponding cells [30-33], suggesting that the abnormal expression of COL3A1 plays an important role in the proliferation and development of various cells. There are also studies found that the expression of COL3A1 in cumulus cells is elevated under the stimulation of follicle stimulating hormone (FSH) [34], and FSH has the function of promoting granulosa cell proliferation [35, 36], suggesting that COL3A1 may affect the proliferation of granulosa cells. Our study showed that COL3A1 expression levels were significantly different in granulosa cells from patients with different ovarian reserve, and down-regulation of COL3A1 significantly inhibited granulosa cell proliferation, indicating that COL3A1 expression is associated with ovarian function, at least in part, by regulating granulosa cell proliferation.

Considering that granulosa cell proliferation was significantly inhibited after overexpression of lnc-GULP1-2:1, and both the expression and localization of COL3A1 can be regulated by lnc-GULP1-2:1, we propose that the inhibition of cell proliferation after overexpression of lnc-GULP1-2:1 may through the increased expression of COL3A1. However, down-regulating the expression level of COL3A1 also significantly inhibited the proliferation of KGN. It seems that the inhibitory effect of lnc-GULP1-2:1 on KGN cell proliferation is at least partly caused by increasing the COL3A1 protein into the nucleus.

Though we found that overexpression of lnc-GULP1-2:1 up-regulated the expression of COL3A1 protein and promoted the entry of COL3A1 protein into the nucleus, but the specific mechanism of how lnc-GULP1-2:1 targets and regulates the expression and localization of COL3A1 is unclear. Therefore, subsequent research should focus on how lnc-GULP1-2:1 participates in the impact on granulosa cell function by regulating COL3A1, such as bioinformatics-based lnc-GULP1-2:1 sequence mutation study, sequencing analysis to analyze the downstream proteins regulated by lnc-GULP1-2:1, to explore the specific mechanism of action that affects the expression and localization of COL3A1.

In our study, we found that knocking down the expression of COL3A1 by siRNA transfection will cause a decrease in the cytoplasmic COL3A1 protein level, thereby affecting cell proliferation, which is consistent with the published reports [30-33]; but the cells overexpress lnc-GULP-2:1 not only up-regulated the protein level of COL3A1, but also promoted the entry of COL3A1 protein into the nucleus, leading to the reduced level of cell proliferation. These results suggest that the expression level of COL3A1 and its subcellular localization in granulosa cells have an important influence on cell function, especially the regulation of cell proliferation. Nevertheless, the specific mechanism of how the expression and localization of COL3A1 in the nucleus and cytoplasm regulate cell proliferation still needs further study and the demonstration of this mechanism will be a more interesting topic for the understanding the development of ovarian physiology and pathology.

Conclusion

In conclusion, this study shows that lnc-GULP1-2:1 can affect the expression level of COL3A1 in cells and increase its localization in the nucleus, thereby affecting the expression level of cell cycle-associated proteins, resulting in the inhibition of granulosa cell proliferation. This study describes its regulation in granulosa cell function from the perspective of lncRNA, provides new ideas and methods for understanding and exploring the mechanism of follicular development and follicular disease, and provides a new strategy for the early diagnosis and treatment of follicular development-related diseases.

Declarations

Ethics approval and consent to participate

This study was approved by the Biomedical Ethics Committee of the First Affiliated Hospital of Zhengzhou University, and all human biologic materials were collected after receiving written informed consent from patients.

Consent for publication

Not applicable.

Availability of data and materials

The data used during this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by grants from the National Natural Science Foundation of China (U1904138 to Guidong Yao, 81820108016 to Yingpu Sun), and the Science and Technology Research Project of Henan Province (172102310396 to Guidong Yao).

Authors' contributions

Y.-P.S. and G.Yao conceived and supervised the project. Y.K., G.Yao., G.Yang. and D.K. performed most of the experiments, Y.X., J.H., Z.X., Y.B., H.F., Q.H. performed some experiments and managed the human sample collection. Y.K. and G.Yao analyzed data and prepared most of the figures. Y.K. and G.Yao wrote the manuscript with the help from the remaining authors.

Acknowledgments

The authors wish to thank all patients from the First Affiliated Hospital of Zhengzhou University for the donation of their biological samples.

References

1. Cabili MN, Trapnell C, Goff L, Koziol M, Tazon-Vega B, Regev A, et al. Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. Genes Dev. 2011;25(18):1915-27. doi:10.1101/gad.17446611.
2. Derrien T, Johnson R, Bussotti G, Tanzer A, Djebali S, Tilgner H, et al. The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. Genome Res. 2012;22(9):1775-89. doi:10.1101/gr.132159.111.
3. Engreitz JM, Ollikainen N, Guttman M. Long non-coding RNAs: spatial amplifiers that control nuclear structure and gene expression. Nat Rev Mol Cell Biol. 2016;17(12):756-70. doi:10.1038/nrm.2016.126.
4. Batista PJ, Chang HY. Long noncoding RNAs: cellular address codes in development and disease. Cell. 2013;152(6):1298-307. doi:10.1016/j.cell.2013.02.012.
5. Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. Nat Rev Genet. 2009;10(3):155-9. doi:10.1038/nrg2521.
6. Li X, Wu Z, Fu X, Han W. lncRNAs: insights into their function and mechanics in underlying disorders. Mutat Res Rev Mutat Res. 2014;762:1-21. doi:10.1016/j.mrrev.2014.04.002.

7. Xiong Y, Liu T, Wang S, Chi H, Chen C, Zheng J. Cyclophosphamide promotes the proliferation inhibition of mouse ovarian granulosa cells and premature ovarian failure by activating the lncRNA-Meg3-p53-p66Shc pathway. Gene. 2017;596:1-8. doi:10.1016/j.gene.2016.10.011.
8. Kimura AP, Yoneda R, Kurihara M, Mayama S, Matsubara S. A Long Noncoding RNA, lncRNA-Amhr2, Plays a Role in Amhr2 Gene Activation in Mouse Ovarian Granulosa Cells. Endocrinology. 2017;158(11):4105-21. doi:10.1210/en.2017-00619.
9. Liu YD, Li Y, Feng SX, Ye DS, Chen X, Zhou XY, et al. Long Noncoding RNAs: Potential Regulators Involved in the Pathogenesis of Polycystic Ovary Syndrome. Endocrinology. 2017;158(11):3890-9. doi:10.1210/en.2017-00605.
10. Silva CA, Yamakami LY, Aikawa NE, Araujo DB, Carvalho JF, Bonfa E. Autoimmune primary ovarian insufficiency. Autoimmun Rev. 2014;13(4-5):427-30. doi:10.1016/j.autrev.2014.01.003.
11. De Vos M, Devroey P, Fauser BC. Primary ovarian insufficiency. Lancet. 2010;376(9744):911-21. doi:10.1016/S0140-6736(10)60355-8.
12. El-Hayek S, Clarke HJ. Control of Oocyte Growth and Development by Intercellular Communication Within the Follicular Niche. Results Probl Cell Differ. 2016;58:191-224. doi:10.1007/978-3-319-31973-5_8.
13. Knight PG, Glister C. TGF-beta superfamily members and ovarian follicle development. Reproduction. 2006;132(2):191-206. doi:10.1530/rep.1.01074.
14. Hull KL, Harvey S. Growth hormone and reproduction: a review of endocrine and autocrine/paracrine interactions. Int J Endocrinol. 2014;2014:234014. doi:10.1155/2014/234014.
15. Brower PT, Schultz RM. Intercellular communication between granulosa cells and mouse oocytes: existence and possible nutritional role during oocyte growth. Dev Biol. 1982;90(1):144-53. doi:10.1016/0012-1606(82)90219-6.
16. Chesnel F, Wigglesworth K, Eppig JJ. Acquisition of meiotic competence by denuded mouse oocytes: participation of somatic-cell product(s) and cAMP. Dev Biol. 1994;161(1):285-95. doi:10.1006/dbio.1994.1028.
17. De La Fuente R, Eppig JJ. Transcriptional activity of the mouse oocyte genome: companion granulosa cells modulate transcription and chromatin remodeling. Dev Biol. 2001;229(1):224-36. doi:10.1006/dbio.2000.9947.
18. Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. Reproduction. 2001;122(6):829-38. doi:10.1530/rep.0.1220829.
19. Yang H, Xie Y, Yang D, Ren D. Oxidative stress-induced apoptosis in granulosa cells involves JNK, p53 and Puma. Oncotarget. 2017;8(15):25310-22. doi:10.18632/oncotarget.15813.
20. Matsuda F, Inoue N, Manabe N, Ohkura S. Follicular growth and atresia in mammalian ovaries: regulation by survival and death of granulosa cells. J Reprod Dev. 2012;58(1):44-50. doi:10.1262/jrd.2011-012.
21. Adeldust H, Zeinoaldini S, Kohram H, Amiri Roudbar M, Daliri Joupari M. In vitro maturation of ovine oocyte in a modified granulosa cells co-culture system and alpha-tocopherol supplementation:

effects on nuclear maturation and cleavage. J Anim Sci Technol. 2015;57:27. doi:10.1186/s40781-015-0061-5.

22. Xu X, Chen X, Zhang X, Liu Y, Wang Z, Wang P, et al. Impaired telomere length and telomerase activity in peripheral blood leukocytes and granulosa cells in patients with biochemical primary ovarian insufficiency. Hum Reprod. 2017;32(1):201-7. doi:10.1093/humrep/dew283.
23. Cho SH, An HJ, Kim KA, Ko JJ, Kim JH, Kim YR, et al. Single nucleotide polymorphisms at miR-146a/196a2 and their primary ovarian insufficiency-related target gene regulation in granulosa cells. PLoS One. 2017;12(8):e0183479. doi:10.1371/journal.pone.0183479.
24. Yao G, He J, Kong Y, Zhai J, Xu Y, Yang G, et al. Transcriptional profiling of long noncoding RNAs and their target transcripts in ovarian cortical tissues from women with normal menstrual cycles and primary ovarian insufficiency. Mol Reprod Dev. 2019;86(7):847-61. doi:10.1002/mrd.23158.
25. Dykes IM, Emanueli C. Transcriptional and Post-transcriptional Gene Regulation by Long Non-coding RNA. Genomics Proteomics Bioinformatics. 2017;15(3):177-86. doi:10.1016/j.gpb.2016.12.005.
26. Rotterdam EA-SPcwg. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). Hum Reprod. 2004;19(1):41-7. doi:10.1093/humrep/deh098.
27. Salaverria I, Royo C, Carvajal-Cuenca A, Clot G, Navarro A, Valera A, et al. CCND2 rearrangements are the most frequent genetic events in cyclin D1(-) mantle cell lymphoma. Blood. 2013;121(8):1394-402. doi:10.1182/blood-2012-08-452284.
28. Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature. 1993;366(6456):704-7. doi:10.1038/366704a0.
29. Kuivaniemi H, Tromp G. Type III collagen (COL3A1): Gene and protein structure, tissue distribution, and associated diseases. Gene. 2019;707:151-71. doi:10.1016/j.gene.2019.05.003.
30. Xu W, Li Z, Zhu X, Xu R, Xu Y. miR-29 Family Inhibits Resistance to Methotrexate and Promotes Cell Apoptosis by Targeting COL3A1 and MCL1 in Osteosarcoma. Med Sci Monit. 2018;24:8812-21. doi:10.12659/MSM.911972.
31. Wang Q, She Y, Bi X, Zhao B, Ruan X, Tan Y. Dexmedetomidine Protects PC12 Cells from Lidocaine-Induced Cytotoxicity Through Downregulation of COL3A1 Mediated by miR-let-7b. DNA Cell Biol. 2017;36(7):518-28. doi:10.1089/dna.2016.3623.
32. Tao R, Fan XX, Yu HJ, Ai G, Zhang HY, Kong HY, et al. MicroRNA-29b-3p prevents Schistosoma japonicum-induced liver fibrosis by targeting COL1A1 and COL3A1. J Cell Biochem. 2018;119(4):3199-209. doi:10.1002/jcb.26475.
33. Gao YF, Zhu T, Chen J, Liu L, Ouyang R. Knockdown of collagen alpha-1(III) inhibits glioma cell proliferation and migration and is regulated by miR128-3p. Oncol Lett. 2018;16(2):1917-23. doi:10.3892/ol.2018.8830.
34. Assou S, Haouzi D, Dechaud H, Gala A, Ferrieres A, Hamamah S. Comparative gene expression profiling in human cumulus cells according to ovarian gonadotropin treatments. Biomed Res Int. 2013;2013:354582. doi:10.1155/2013/354582.

35. Navalakhe RM, Jagtap DD, Nayak SU, Nandedkar TD, Mahale SD. Effect of FSH receptor-binding inhibitor-8 on FSH-mediated granulosa cell signaling and proliferation. Chem Biol Drug Des. 2013;82(2):178-88. doi:10.1111/cbdd.12149.

36. Hernandez-Coronado CG, Guzman A, Rodriguez A, Mondragon JA, Romano MC, Gutierrez CG, et al. Sphingosine-1-phosphate, regulated by FSH and VEGF, stimulates granulosa cell proliferation. Gen Comp Endocrinol. 2016;236:1-8. doi:10.1016/j.ygcen.2016.06.029.

Figures

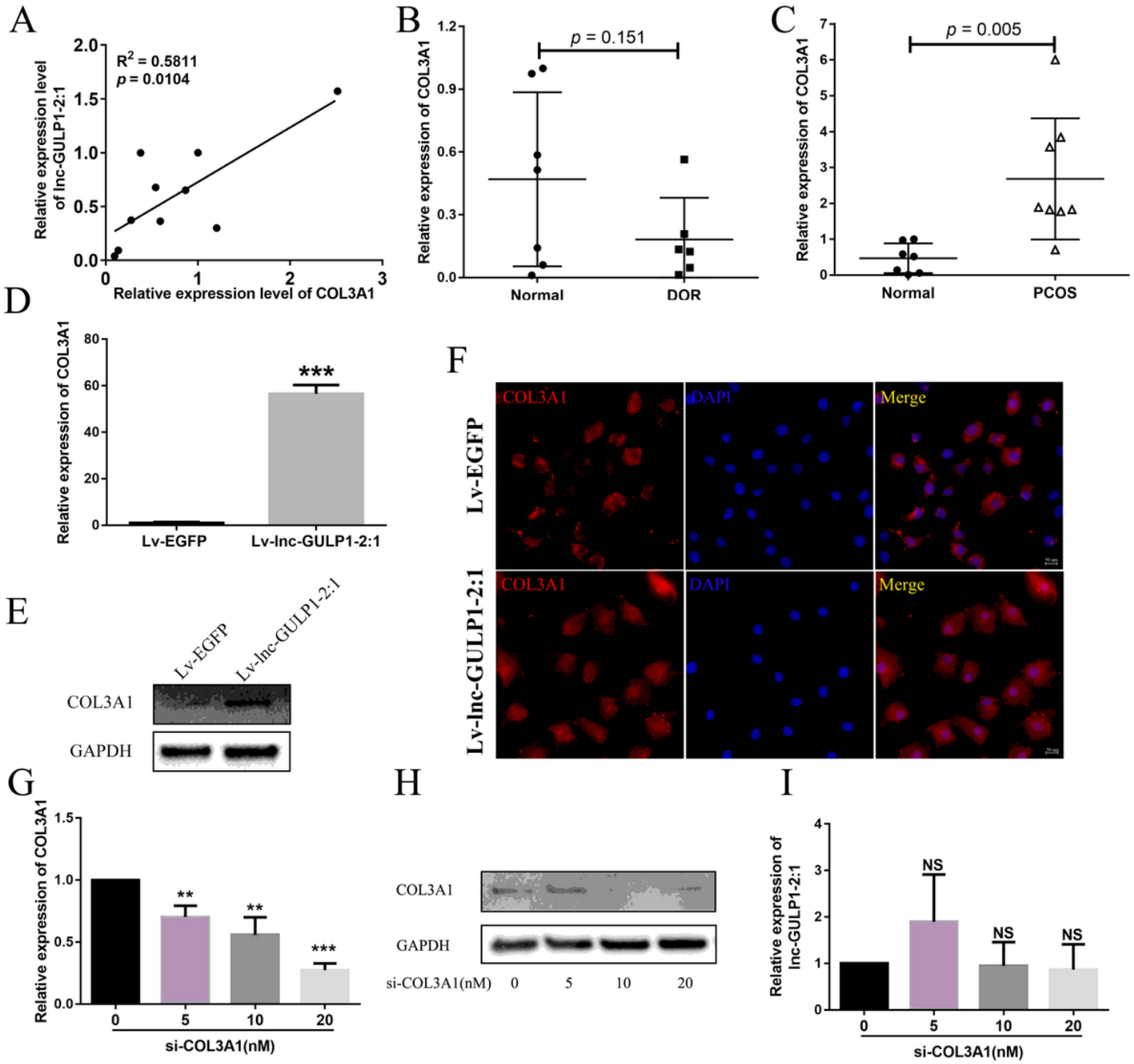

Figure 1

Lnc-GULP1-2:1 overexpression affects the expression and localization of COL3A1 in granulosa cells. The expression correlation of lnc-GULP1-2:1 and COL3A1 was analyzed by real-time PCR in different cell lines (including Bewo, HTR-8/SVneo, OVCAR3, SKOV3, MDA-MB-231, Hep G2, 293T, U87 MG and JAR) (A). The expression of COL3A1 from patient of normal, DOR and PCOS-derived granulosa cells (B & C). The effect of overexpression of lnc-GULP1-2:1 on COL3A1 mRNA and protein expression (D & E). The expression and localization of COL3A1 in KGN cell lines analyzed by immunofluorescence assay (F). Red signal, COL3A1 signal. Blue signal, DAPI signal indicates nuclear localization. Bar = 50 μm. The mRNA and protein expression of COL3A1 can be dose-dependently reduced by using COL3A1 specific siRNA (G & H). Knocking down of COL3A1 on the expression of lnc-GULP1-2:1 (I). GAPDH was used as an internal control. NS indicates no significant difference, * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.0001$.

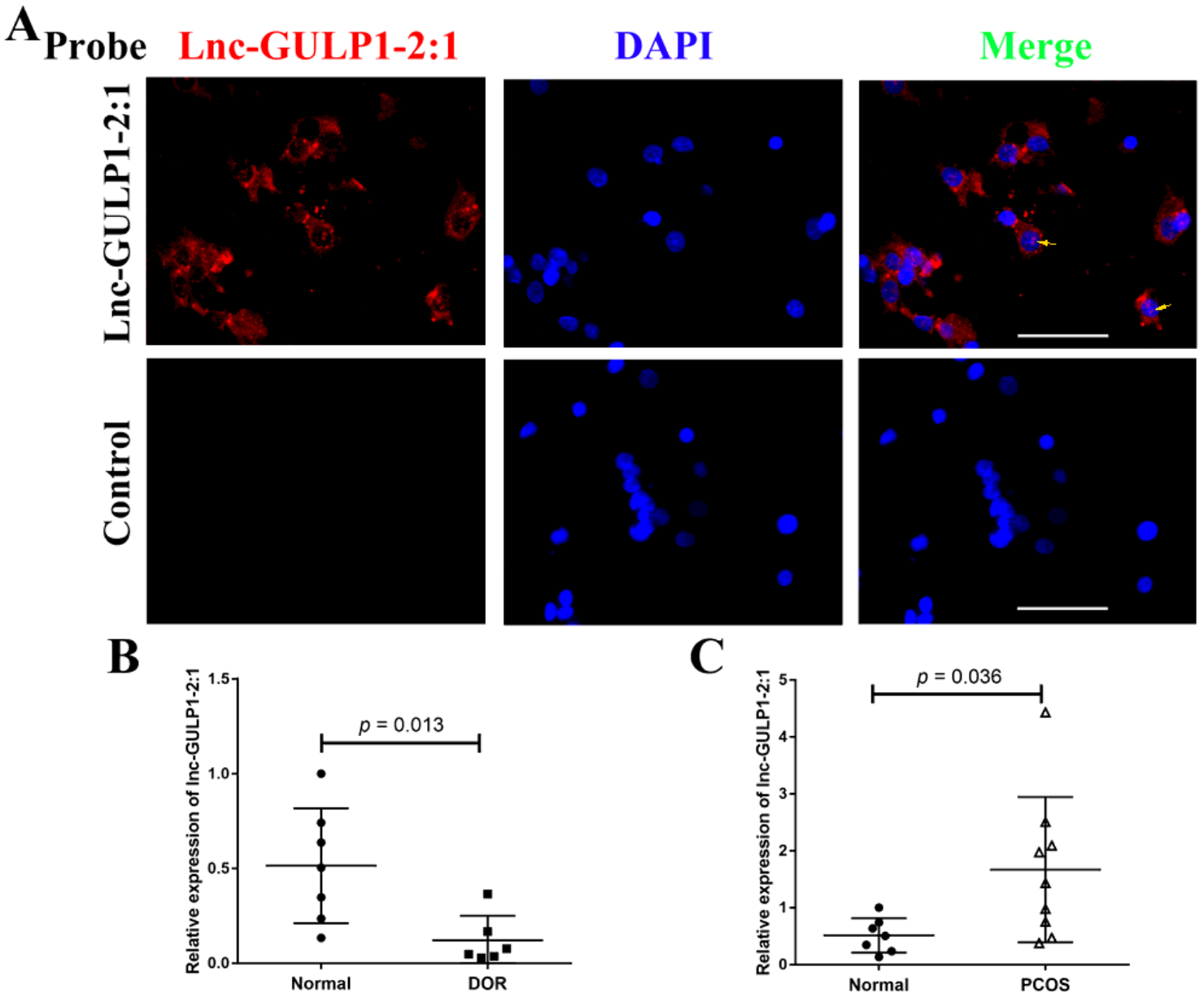

Figure 2

The localization and expression of lnc-GULP1-2:1 in granulosa cells. In situ hybridization was used to detect the expression of lnc-GULP1-2:1 in human primary granulosa cells (A). Red signal, lnc-GULP1-2:1 expression. Blue signal, DAPI signal indicates nuclear localization. The yellow arrow in the merge graph shows the punctate expression of lnc-GULP1-2:1 signal in the nucleus. Bar = 50 μm. The expression of lnc-GULP1-2:1 in granulosa cells derived from normal, DOR and PCOS patient were analyzed by using real-time PCR (B & C). The GAPDH gene was used as an internal reference. DOR, diminished ovarian reserve. PCOS, polycystic ovary syndrome. $p < 0.05$ indicates the significant difference.

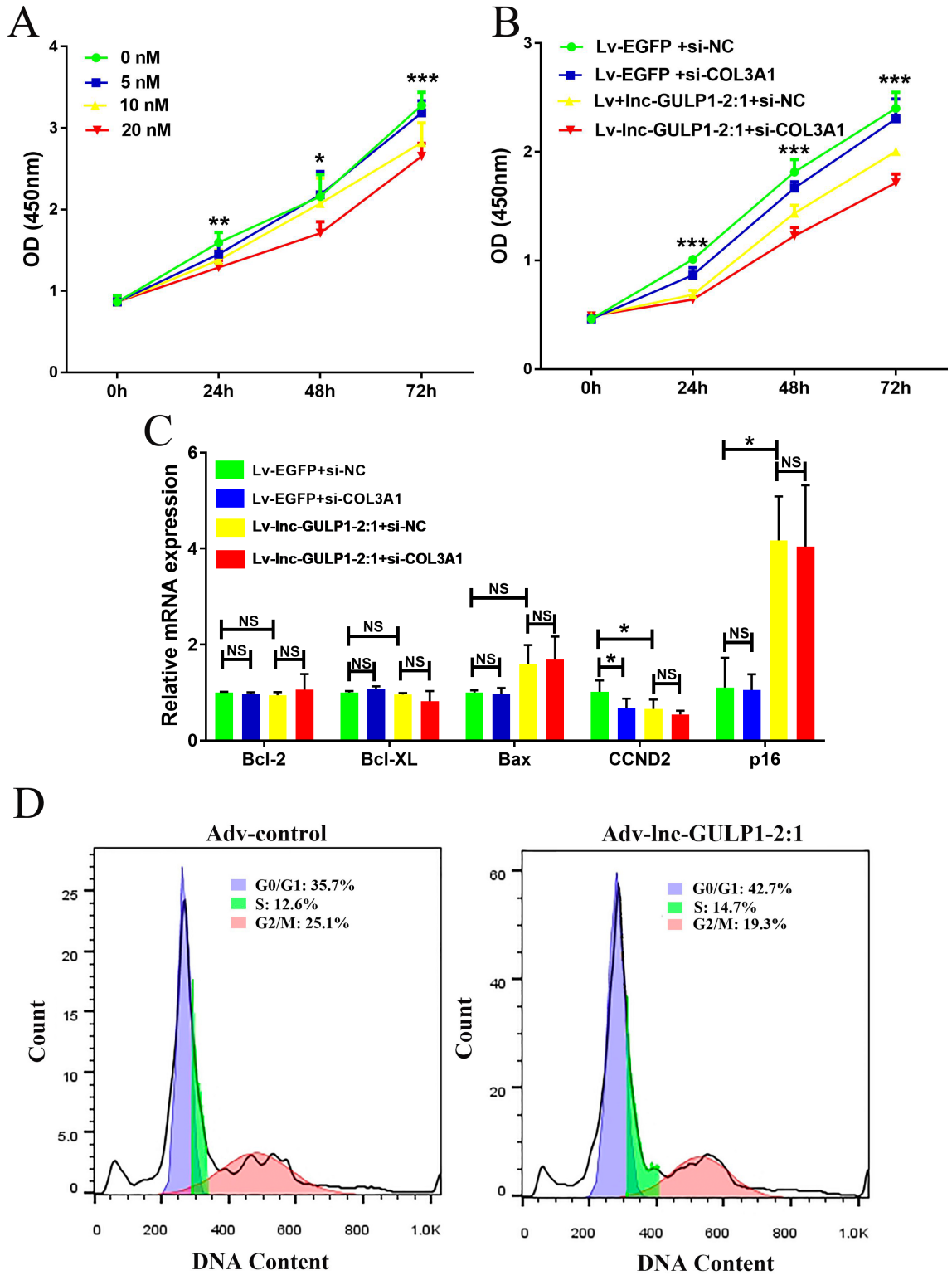

Figure 3

Effect of COL3A1 expression on granulosa cell proliferation and related gene expression. KGN cell proliferation was inhibited by knocking down of COL3A1 expression in dose-dependent manner (A). Effects of simultaneous silencing of COL3A1 expression on cell proliferation in Lv-lnc-GULP1-2:1 and Lv-EGFP cells (B). The expression of Bcl-2, Bcl-XL, Bax, CCND2 and p16 in Lv-lnc-GULP1-2:1 and Lv-EGFP cells by knockdown of COL3A1 (C). GAPDH was used as an internal control. The concentration of siRNA in plots B and C is 20 nM. NC, negative control. si-NC, silence control. NS indicates no significant difference, * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.0001$. Primary granulosa cells were infected with 5×1010 PFU/ml adenovirus overexpress lnc-GULP1-2:1 (adv-lnc-GULP1-2:1) or its control (adv-control), and flow cytometry was used to detect cell cycle changes on cells treated with adenovirus after 48 hours (D).

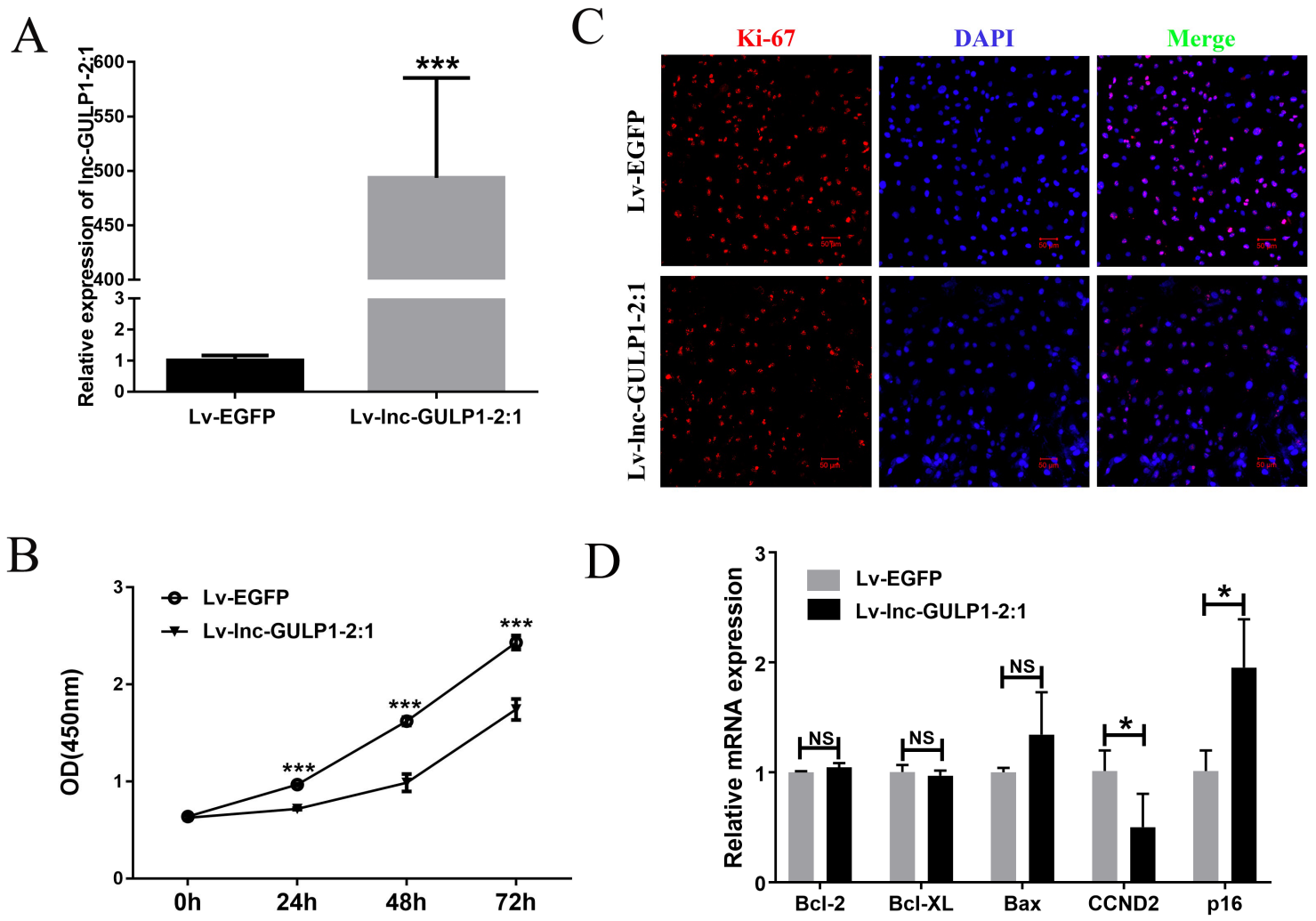

Figure 4

. Effects of lnc-GULP1-2:1 on granulosa cell proliferation and related gene expression. Lv-lnc-GULP1-2:1 capable of overexpressing lnc-GULP1-2:1 (A). Cell proliferation was analyzed in group of Lv-EGFP and Lv-lnc-GULP1-2:1 at 0 h, 24 h, 48 h and 72 h by using CCK-8 (B). “0 h” means the beginning of cell attachment. The Ki-67 expression in KGN cell lines was analyzed by using immunofluorescence assay

(C). Red signal, Ki-67. Blue signal, DAPI signal indicates nuclear localization. Bar = 50 μm. Real time PCR was used to detect the expression of genes related to cell proliferation, cell apoptosis and cell cycle (D). The GAPDH gene was used as an internal reference gene. Lv-EGFP□KGN control cell line overexpressing EGFP□Lv-lnc-GULP1-2:1, KGN cell line overexpressing lnc-GULP1-2:1. NS indicates no significant difference, * indicates $p < 0.05$, and*** indicates $p < 0.0001$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterial.docx
- SupplementaryTable1.docx