

Lnc-GULP1-2:1 Affects Granulosa Cell Proliferation by Regulating COL3A1 Expression and Localization

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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. Silencing COL3A1 gene in KGN cells also significantly inhibited cell proliferation, which may be related to the regulation of CCND2 gene expression.

Conclusions: This study demonstrates that lnc-GULP1-2:1 may participate in the regulation of granulosa cell proliferation by regulating the expression and localization of COL3A1 protein, which 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 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[11] are all could be the causes of POI. 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.

Granulosa cells play important roles in follicular development and oocyte maturation. The proliferation, differentiation and apoptosis of granulosa cells are under the control of various hormones and cytokines at different developmental stages of follicles, which have an irreplaceable regulatory effect on follicular development[12]. Oocyte growth and development are supported by surrounding granulosa cells[13], the meiosis[14] and global transcriptional activity of the oocyte genome[15] are also regulated by granulosa cells. In turn, granulosa cell proliferation and differentiation[16] are also regulated by oocyte. Abnormal states of granulosa cells were observed in many ovarian-related diseases, and the apoptosis of ovarian granulosa cells was observed at different levels, such as molecular, gene and tissue level in many studies related to POI.

In our previous study, next generation sequencing revealed that lnc-GULP1-2:1 and COL3A1 were significantly down-regulated in ovarian cortical tissue in patients with POI than normal ovarian cortical tissue [17]. Co-expression network analysis showed that COL3A1 was the *cis*-predicted transcript of lnc-GULP1-2:1[4, 18]. 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 [17]. 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.

In this study, we found that overexpression of lnc-GULP1-2:1 in KGN cells up-regulated the expression of COL3A1 and promoted migration of COL3A1 protein into the nucleus. This is the first report that lncRNA affects the proliferation of human granulosa cells by regulating COL3A1.

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[17]; and the included PCOS patients were younger than 35 years old and were diagnosed according to the Rotterdam 2003 criteria[19], 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 the first transvaginal ultrasound guided follicular punctures, and without history of ovarian surgery.

Primary luteinized granulosa cell isolation and cell culture

Density gradient centrifugation (Lymphocyte Separation Medium, LTS1077, Tianjin, China) was used for the extraction of luteinized granulosa cells from follicular fluid of patients undergoing transvaginal ultrasound guided follicular punctures. 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[17]. KGN, MDA-AB-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 cells were cultured in MEM (Gibco). WM1382 and JAR were cultured in RPMI-1640 medium (Invitrogen). All cell lines were routinely cultured at 37°C in an atmosphere of 5% CO₂ 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 ddH₂O) was incubated for 5 min at 25°C and 20 min at 46°C, incubated for 1 min at 95 °C and then maintained at 4°C. For real-time PCR, all reactions were performed in triplicate with iTaq™ Universal SYBR® Green Supermix (Bio-Rad) under the following conditions: 30 s at 95°C for initial denaturation, followed by 40 cycles of segments of 95 °C for 3 s and 60 °C for 30 s in 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal reference gene. Gene expression was calculated by using the method of $2^{-\Delta\Delta Ct}$, $\Delta Ct = \Delta Ct_{\text{target}} - \Delta Ct_{\text{reference}}$, $-\Delta\Delta Ct = -(\Delta Ct_{\text{sample}} - \Delta Ct_{\text{control}})$. 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 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°C 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°C 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 Inc-GULP1-2:1 (Lv-Inc-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% CO₂ at high humidity. Cells were treated with 4% paraformaldehyde for 20 min, then gently washed with 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) at 4°C 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 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% CO₂ at high humidity, then treated with 4% paraformaldehyde for 20 min. After washing the coverslips, cells were digested by 20 µg/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°C. Next, the prehybridization solution was discarded, and a hybridization solution (Servicebio)

containing Inc-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/ μ l, was added dropwise, and hybridization was carried out at 37°C 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°C for 50 min, and then washed 3 \times 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.

Statistical analysis

Data were expressed as mean \pm 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 Inc-GULP1-2:1 in luteinized granulosa cells is correlated with ovarian function status

In our recent study, ovarian cortical tissues from women with normal menstrual cycles and POI patients who underwent IVA surgery were sequenced for the analysis of non-coding RNA and mRNA expression, and we found that Inc-GULP1-2:1 is a differentially expressed lncRNA and may be closely related to ovarian follicular development[17]. In this study, we focused on the function of Inc-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 Inc-GULP1-2:1 is mainly localized in the cytoplasm of luteinized granulosa cells by using fluorescence *in situ* hybridization. It is suggested that Inc-GULP1-2:1 mainly plays a role in post-transcriptional regulation. However, Inc-GULP1-2:1 punctate expression signals can also be seen in the nucleus of some cells (Fig. 1A), indicating its involvement in the regulation of gene expression regulation.

Further, we collected luteinized granulosa cells from DOR and PCOS patients to explore whether the expression of Inc-GULP1-2:1 in luteinized granulosa cells is related to different ovarian function status. The results showed that the expression of Inc-GULP1-2:1 in luteinized granulosa cells of DOR patients was significantly lower than that of luteinized granulosa cells from normal patients ($p < 0.05$) (Fig. 1B), while the expression of Inc-GULP1-2:1 in luteinized granulosa cells of PCOS was significantly higher than that of luteinized granulosa cells from normal patients ($p < 0.05$) (Fig. 1C). These results indicate that Inc-GULP1-2:1 expression level is different in luteinized granulosa cells from different patient with various ovarian function, suggesting that it may be involved in ovarian follicular development.

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

To further evaluate the functional role of Inc-GULP1-2:1 in granulosa cells, lentivirus-stabilized KGN cell line capable of overexpressing Inc-GULP1-2:1 (Lv-Inc-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 Inc-GULP1-2:1 was successfully constructed (Fig. 2A). We know that granulosa cell proliferation is essential for follicular development, so cell counting kit-8 (CCK-8) was used to analyze the effect of Lv-Inc-GULP1-2:1 expression on KGN cell proliferation. The results revealed that granulosa cell proliferation was significantly inhibited in Inc-GULP1-2:1 overexpressed KGN cells (Fig. 2B). Genes related to cell proliferation, apoptosis and cell cycle were also analyzed, and the results showed that overexpression of Inc-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 was up-regulated but with no significant difference ($p > 0.05$). However, the expression of the cell cycle-related genes was changed significantly, with the expression of CCND2 was significantly down-regulated, while p16 gene expression was significantly up-regulated (Fig. 2C). These results suggested that cell proliferation regulated by overexpression of Inc-GULP1-2:1 may be involved in the regulation of cell cycle progression.

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

As we pointed out in our recent published paper, COL3A1 is the *cis* target of Inc-GULP1-2:1[17]. In order to further verify the regulatory relationship between Inc-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 Inc-GULP1-2:1 and COL3A1 ($R^2 = 0.5811$, $p < 0.05$) (Fig. 3A), indicating that there may be a regulatory relationship between the two genes. 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 although the expression of COL3A1 in DOR patient-derived luteinized granulosa cells was not significantly different from the normal group, the expression level in DOR was lower than that in the normal group (Fig. 3B); and the expression of COL3A1 was significantly

increased in PCOS patient-derived luteinized granulosa cells (Fig. 3C). These results showed that the expression of COL3A1 in luteinized granulosa cells from normal, DOR and PCOS patients were consistent with that of lnc-GULP1-2:1, indicating that there is a regulatory relationship between lnc-GULP1-2:1 and COL3A1 expression in human luteinized granulosa cells, and this regulatory relationship may be associated with follicular development and ovarian function.

Considering the expression correlation between lnc-GULP1-2:1 and COL3A1, and bioinformatics predictions revealed that COL3A1 is a potential target gene of lnc-GULP1-2:1. Therefore, a very important question is how does lnc-GULP1-2:1 regulate COL3A1? Consistent with our previous results, lnc-GULP1-2:1 overexpression significantly upregulated both mRNA and protein levels of COL3A1 (Fig. 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 (Fig. 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, will the change of COL3A1 expression level affect the expression of lnc-GULP1-2:1? By using COL3A1 specific siRNA to knockdown both the mRNA and protein expression of COL3A1 in KGN cells (Fig. 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 (Fig. 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. Based on this, will the down-regulation of COL3A1 promote KGN cell proliferation? However, the results were contrary to our expectations. By knocking down the expression of COL3A1 gene in KGN cells, cell proliferation was not increased, but inhibited significantly in a dose-dependent manner (Fig. 4A). Furthermore, simultaneous down-regulation of COL3A1 levels in Lv-lnc-GULP1-2:1 and Lv-EGFP cells further inhibited cell proliferation (Fig. 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 cells. 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 (Fig. 4C). These results indicate that lnc-GULP1-2:1 mainly causes the increased localization of COL3A1 in the nucleus, thereby inhibiting granulosa cell proliferation. However, silencing of the COL3A1 gene results in down-regulation of total COL3A1 protein levels, which may affect the protein level of extracellular COL3A1 and thus inhibit granulosa cell proliferation. Therefore, the down-regulation of COL3A1 in Lv-lnc-GULP1-2:1 cell inhibits cell proliferation in two ways. On the one hand, downregulation of total COL3A1 expression inhibits cell proliferation due to the lacking of COL3A1

protein in cells; On the other hand, intracellular elevated lnc-GULP1-2:1 promotes COL3A1 protein entry into the nucleus, thereby inhibiting cell proliferation due to the abnormal localization of COL3A1 protein. In addition, down-regulation of COL3A1 had no significant effect on Bcl-2, Bcl-XL, Bax and p16 genes, but down-regulated CCND2 gene expression.

Discussion

During follicular development, granulosa cells surround the oocyte and differentiate into mural granulosa cells and cumulus granulosa cells[20]. The physiological function of granulosa cells depends on paracrine and autocrine cytokines in the ovarian microenvironment and reproductive hormones in peripheral blood[21]. Oocytes and granulosa cells grow and develop in a highly coordinated, interdependent manner. Granulosa cells provide nutrient and maturation-related factors to oocytes, ensuring oocyte development and maturation[22]. 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[23], and eventually result in a decrease in the number of follicles[24]. 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[25, 26].

In this study, we found that lnc-GULP1-2:1 was not only expressed low in the ovarian cortical tissues of POI patients, but also in luteinized granulosa cells of patients with DOR. It indicates that lnc-GULP1-2:1 may have a specific relationship in granulosa cell proliferation and ovarian reserve *in vivo*. *In situ* hybridization revealed that lnc-GULP1-2:1 mainly localized in the cytoplasm of luteinized granulosa cell, suggesting that it may act mainly through post-transcriptional regulation. However, we also found that lnc-GULP1-2:1 has a small amount of localization in the nucleus, indicating that it may also play a role 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 CDK4 or 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]. In this study, we found that upregulation of lnc-GULP1-2:1 significantly decreased the expression of cell cycle-associated gene CCND2, while p16 expression was significantly up-regulated, indicating 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 found that lnc-GULP1-2:1 affects the gene expression and protein localization of COL3A1. COL3A1 gene encodes the pro- α 1 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], indicating 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 FSH[34], and FSH has the function of promoting granulosa cell proliferation[35, 36], indicating 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, suggesting 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. But the results were not as we expected. When silencing the expression level of COL3A1, the proliferation of KGN was also inhibited. Down-regulation of COL3A1 significantly inhibited the expression of CCND2, while down-regulation of COL3A1 in lnc-GULP1-2:1 overexpressed KGN cell further inhibited the expression of CCND2, but down-regulation of COL3A1 did not affect the expression of p16 gene. These results indicate that lnc-GULP1-2:1 may increase the protein localization of COL3A1 in the nucleus, thus inhibit granulosa cell proliferation; and the total protein level of COL3A1 is also important in cell proliferation, with the silencing of COL3A1 also inhibit granulosa cell proliferation. These results also indicate that the expression and localization of COL3A1 is very important for granulosa cell proliferation.

However, there are also some limitations in this study. The normal group of patients included in this study was younger than 35 years old, while the DOR group included secondary infertility patients older than 40 years old. Therefore, our study cannot exclude that the decrease in the expression level of lnc-GULP1-2:1 in the DOR group is caused by age factor. In view of this, further studies should compare the expression of lnc-GULP1-2:1 between the two groups by including DOR patients younger than 35 years old and normal group patients.

In this study, 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 addition, 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-GULP1-2:1 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. As for how this change of COL3A1 protein in the cytoplasm and nucleus affects the proliferation of granulosa cells, we have no clear experimental conclusions. However, from the results of lnc-GULP-2:1 and COL3A1 affecting cell cycle related genes, the large number of COL3A1 into the nucleus may cause it to bind to some proteins in the nucleus and thus affect the expression of genes related to cell proliferation, such as CCND2 or p16 genes etc. These results indicate 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.

In summary, this study shows that lnc-GULP1-2:1 can affect the expression level of COL3A1 in cells and increase the 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

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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.

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Availability of data and materials

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

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.

Competing interests

The authors declare that they have no competing interests.

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Figures

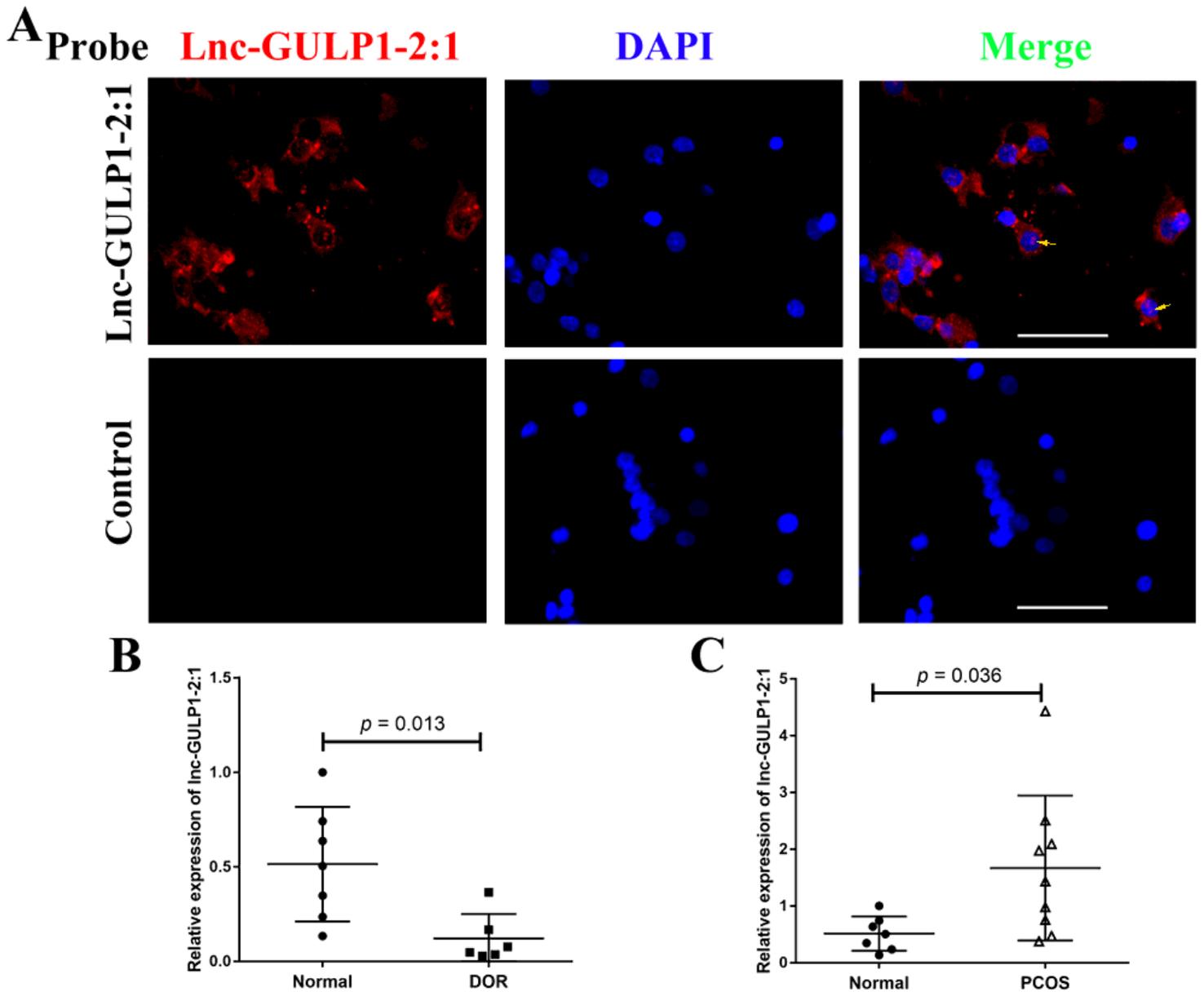


Figure 1

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 μ m. 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.

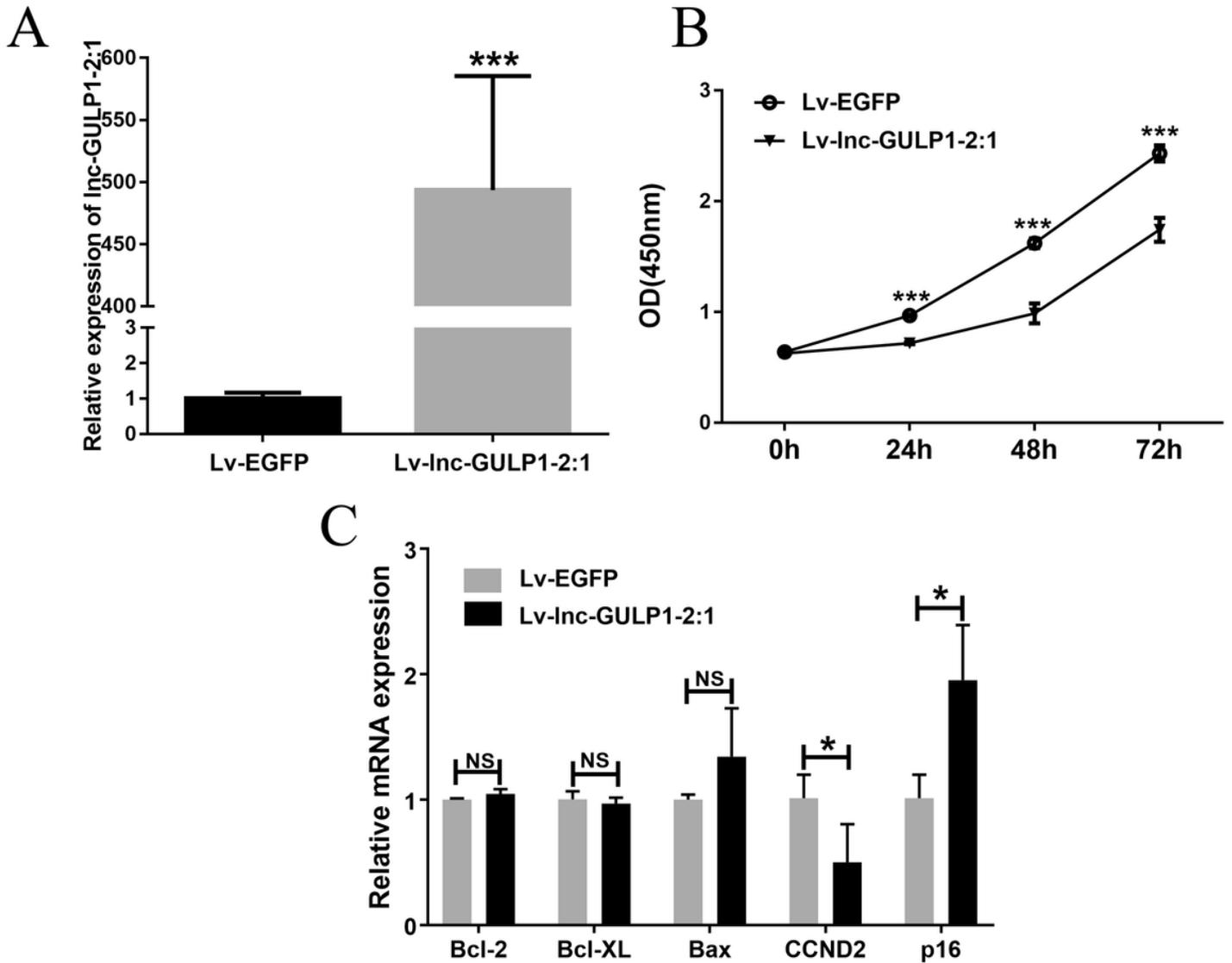


Figure 2

Effects of Inc-GULP1-2:1 on granulosa cell proliferation and related gene expression. Lv-Inc-GULP1-2:1 capable of overexpressing Inc-GULP1-2:1 (A). Cell proliferation was analyzed in group of Lv-EGFP and Lv-Inc-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. Real time PCR was used to detect the expression of genes related to cell proliferation, cell apoptosis and cell cycle (C). The GAPDH gene was used as an internal reference gene. Lv-EGFP/KGN control cell line overexpressing EGFP/Lv-Inc-GULP1-2:1, KGN cell line overexpressing Inc-GULP1-2:1. NS indicates no significant difference, * indicates $p < 0.05$, and *** indicates $p < 0.0001$.

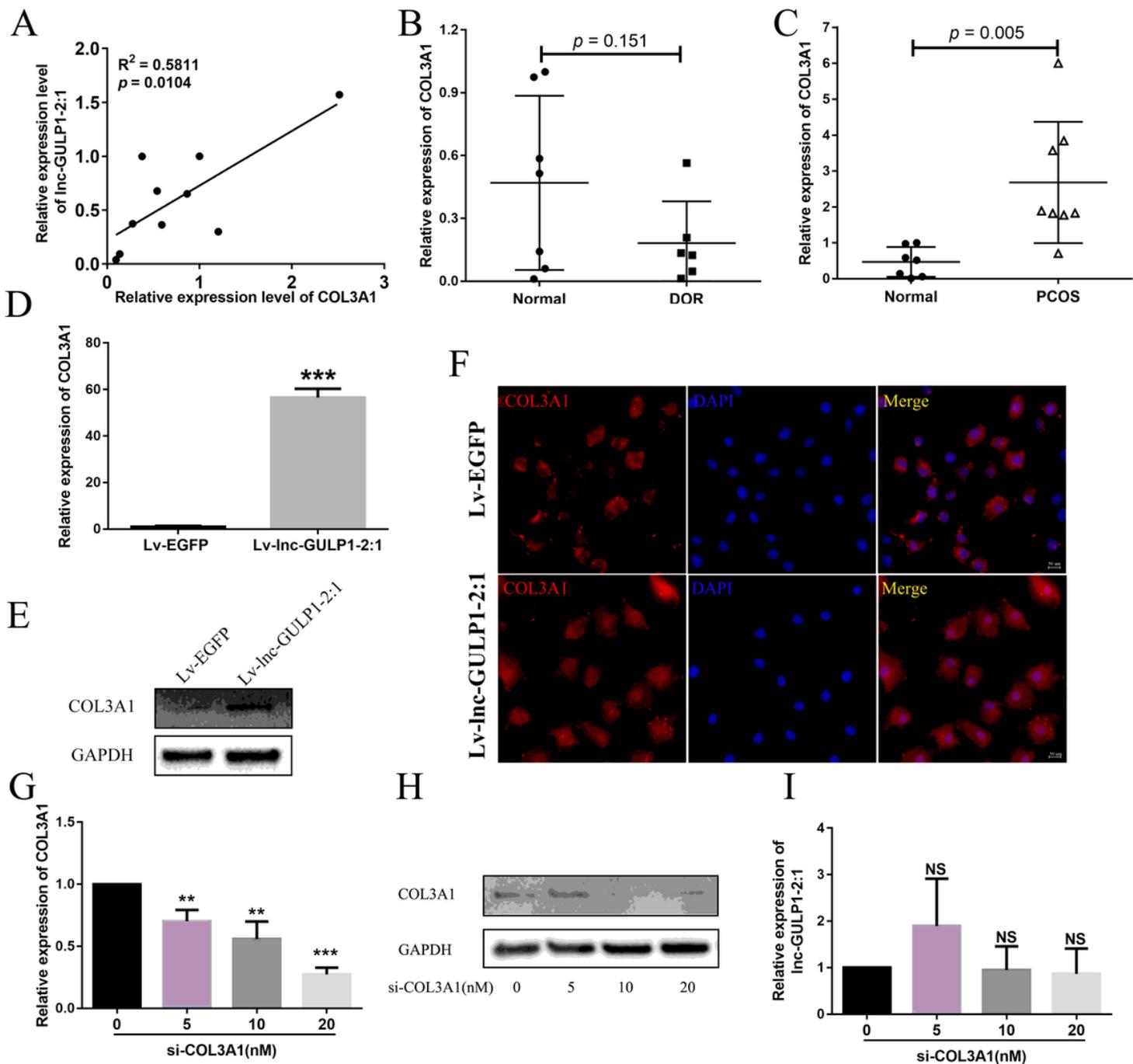


Figure 3

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-AB-231, HepG2, 293T, U87, WM1382 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 μ m. 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 $p < 0.0001$.

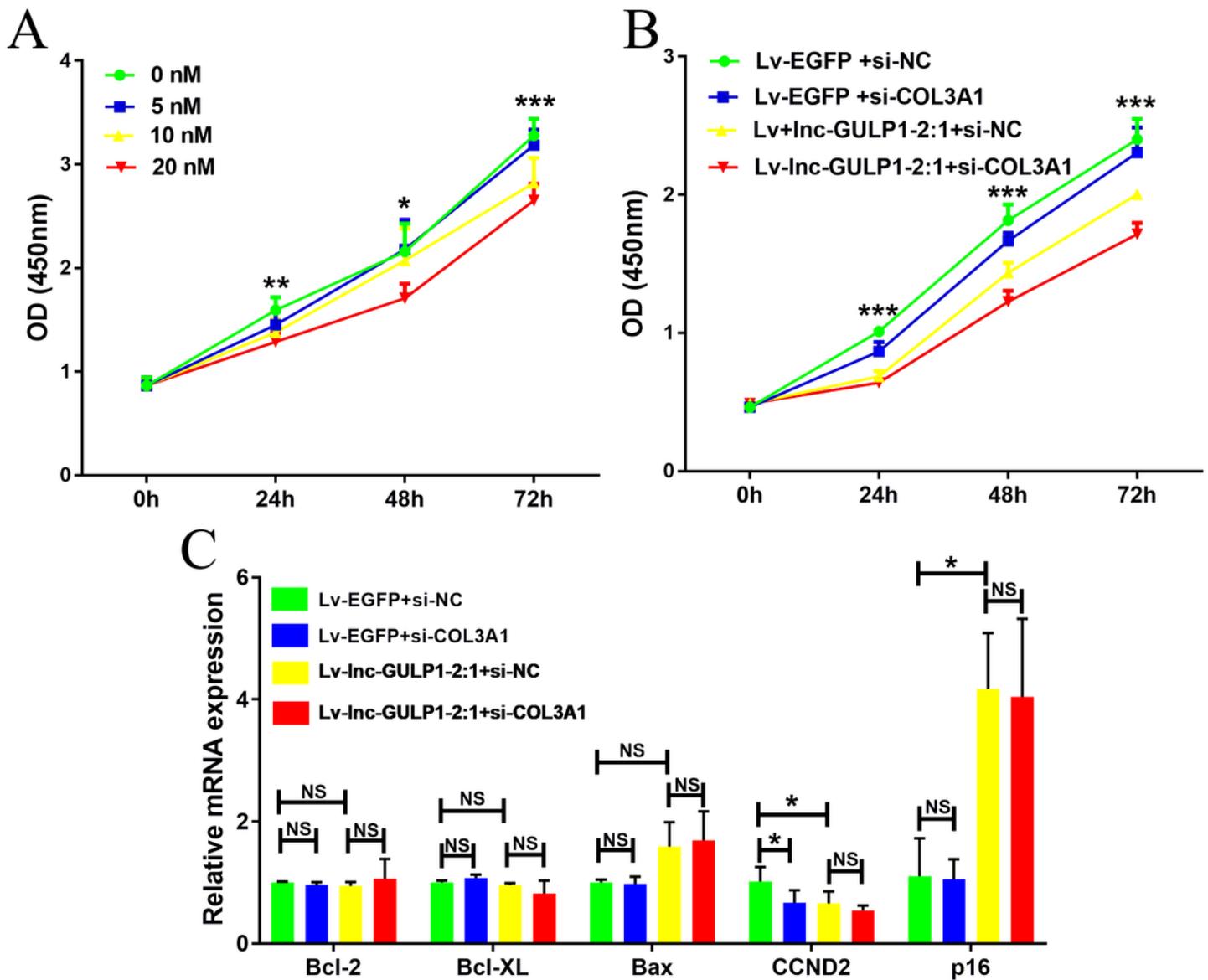


Figure 4

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 $p < 0.0001$.

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