Down-regulation of long non-coding RNA *MALAT1* inhibits granulosa cell proliferation in endometriosis by up-regulating P21 via activation of the ERK/MAPK pathway

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Running title: *MALAT1* long non-coding RNA and endometriosis

Abstract

STUDY QUESTION: Is there a specific mechanism underlying the association between lung adenocarcinoma transcript 1 (*MALAT1*) and endometriosis-related infertility?

SUMMARY ANSWER: The down-regulation of *MALAT1* in endometriosis granulosa cells (GCs) may have an adverse effect on the growth and development of oocytes by inhibiting GC proliferation, due to cell cycle-dependent mechanisms that enhance P21 expression through activation of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway.

WHAT IS KNOWN ALREADY: The association between endometriosis and infertility is well supported throughout the literature, and endometriosis *per se* and its surgical treatment have an adverse effect on the ovarian reserve and on oocyte development. *MALAT1*, one of the most extensively expressed and evolutionarily conserved transcripts, has been implicated to play a role in human development and many diseases. However, little is known about the role of *MALAT1* long non-coding RNA (lncRNA) in endometriosis and its associated infertility.

STUDY DESIGN, SIZE, DURATION: We measured *MALAT1* lncRNA expression levels in GCs from 52 endometriosis patients and 52 controls. Also, *MALAT1* was knocked down in a human GC tumor-derived cell line, KGN, to investigate the role of *MALAT1* and its molecular mechanism in cell proliferation.

PARTICIPANTS/MATERIALS, SETTING, METHODS: GCs were collected from women with or without endometriosis undergoing IVF or ICSI treatment. All endometriosis patients were diagnosed by laparoscopy or laparotomy, and control patients were limited to male factor or tubal disease and had a normal ovarian reserve. Quantitative real-time PCR (qRT-PCR) was used to measure the differential expression levels of *MALAT1* lncRNA between endometriosis patients and controls. The receiver operating characteristic (ROC) curve was drawn to evaluate the diagnostic values of *MALAT1* in endometriosis. In the KGN cell line, *MALAT1* was knocked down with locked nucleic acid GapmeRs. Cell counting kit-8 assays, ethynyl-2-deoxyuridine assays and flow cytometry were used to study the role of *MALAT1* in cell proliferation and cell-cycle progression, and western blotting was performed to detect the potential underlying mechanism.

MAIN RESULTS AND THE ROLE OF CHANCE: We first found that *MALAT1* lncRNA was significantly down-regulated in endometriosis GCs and was associated with the antral follicle count ($R = 0.376$, $P < 0.001$ versus control). In addition, *MALAT1* lncRNA levels were significantly lower in the GCs of infertile women with advanced stages of endometriosis ($P = 0.01$ versus control). The ROC curves illustrated strong separation between all the endometriosis patients and the control group (AUC: 0.705; 95% CI: 0.606–0.804; $P < 0.001$), Stage I-II and control group (AUC: 0.651; 95% CI: 0.536–0.767; $P = 0.016$), and Stage III-IV and control group (AUC: 0.827; 95% CI: 0.718–0.936; $P < 0.001$). *MALAT1* lncRNA was primarily localized in the nuclei of GCs. We found a negative correlation between *MALAT1*

lncRNA and *P21* mRNA in the GCs from patients (R = –0.628; P < 0.001). *MALAT1* knockdown in KGN cells inhibited cell proliferation and cell-cycle progression. In addition, *MALAT1* knockdown induced an increase in both the mRNA and protein levels of P21, and of P53, phosphorylated ERK1/2 (p-ERK1/2) and phosphorylated c-Jun N-terminal protein kinase (p-JNK) protein levels, as well as causing a decrease in cyclin dependent kinase 2 (CDK2), cyclin D1 and p-P38 MAPK protein levels. Furthermore, inhibition of the ERK/MAPK pathway with U0126, the up-regulation of p-ERK1/2, P21 and P53, and the down-regulation of CDK2 and cyclin D1 by the knockdown of *MALAT1* were all attenuated by *MALAT1* knockdown. Therefore, *MALAT1* may regulate GC proliferation through P21/P53-dependent control of the cell cycle, and the ERK/MAPK pathway participates in this process.

LARGE SCALE DATA: None.

LIMITATIONS, REASONS FOR CAUTION: The hormonal treatment used in IVF and surgical removal of endometriotic lesions may have altered *MALAT1* expression in GCs. The ovarian granulosa-like tumor cell line, KGN, was used for further functional and mechanistic studies due to the difficulties in obtaining human GCs in sizable amounts and maintaining primary cultures.

WIDER IMPLICATIONS OF THE FINDINGS: Our finding represents the first example of an lncRNA-based mechanism in endometriosis GCs. Women with endometriosis show altered *MALAT1* expression levels in GCs that may impair fertility by regulating the function of GCs. Therefore, analysis of *MALAT1* and its

molecular mechanisms of action provide new insights into the pathogenesis of endometriosis and its associated infertility.

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Keywords: *MALAT1*; long non-coding RNA; endometriosis; infertility; granulosa cells; P21; P53; extracellular signal-regulated kinase; mitogen-activated protein kinase; proliferation.

Introduction

Endometriosis is a common gynecological disease with a highly enigmatic etiopathogenesis affecting 10–15% of women of reproductive age (Olive and Pritts, 2001), and it is responsible for dysmenorrhea, pelvic pain and infertility. Endometriosis has a prevalence of 25–50% in women with infertility and 30–50% of women with endometriosis are infertile (Missmer *et al.* , 2004). Although the association between endometriosis and infertility is well supported throughout the literature, the exact underlying mechanisms are still unknown. De Ziegler *et al* (de Ziegler *et al.* , 2010*)* proposed that endometriosis-related infertility may result from a distorted pelvic anatomy, a hostile peritoneal environment, an altered endometrial receptivity or a diminished ovarian reserve, or a combination of those. Endometriosis

per se and its surgical treatment have an adverse effect on the ovarian reserve and oocyte development. The ovarian follicular microenvironment and maternal signals, mediated mainly by granulosa cells (GCs) and cumulus cells (CCs), are responsible for folliculogenesis and oocyte growth and maturation (Coticchio *et al.* , 2015). Thus we have investigated the effect of endometriosis on GCs to identify a novel pathogenesis of endometriosis-related subfertility.

A large fraction of the human genome is transcribed to produce large numbers of non-coding RNAs (ncRNAs), including microRNAs (miRNAs), siRNAs, piwi-interacting RNAs (piRNAs) and long non-coding RNAs (lncRNAs), with only less than 3% of the human genome being directly implicated in protein coding (Djebali *et al.* , 2012, Hangauer *et al.* , 2013). Among these, lncRNAs have become an important scientific research area. LncRNAs are defined as transcripts longer than 200 nucleotides in length and are involved in the regulation of gene expression. LncRNAs have distinct biological functions via different molecular mechanisms, including functions in X-chromosome inactivation (Brown *et al.* , 1991), imprinting (Brannan *et al.* , 1990), trans-acting gene regulation (Rinn *et al.* , 2007) and the regulation of nuclear import (Willingham *et al.* , 2005). Increasing evidence indicates that lncRNAs play important roles in gynecological diseases, and they could potentially serve as vital regulators in the progression of these diseases. These lncRNAs include HLA complex group 26 (*HCG26)* (Liu *et al.* , 2017*)*, nuclear paraspeckle assembly transcript 1 (*NEAT1)* (Chai *et al.* , 2016) and metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) (Li *et al.* , 2016).

However, little is known about the role of lncRNAs in endometriosis. Sun *et al.* (Sun *et al.* , 2014) first reported the lncRNA expression patterns in human ectopic and eutopic endometrial tissue. Although thousands of lncRNAs have been identified, only a few lncRNAs have been functionally characterized, such as lncRNA *H19*, which represents the first example of an lncRNA involved in women with endometriosis and infertility (Ghazal *et al.* , 2015). *H19* is one of the most highly abundant and conserved transcripts involved in the mammalian development and tumorigenesis, and highly conserved sequences are commonly known to exert important physiological functions.

Another well-characterized lncRNA that is highly evolutionarily conserved and is extensively expressed in mammalian cells is *MALAT1* (Ma *et al.* , 2015). According to our previous microarray data analysis (GSE95728), *MALAT1* is also highly expressed in the human GCs collected from women with tubal disease or male factor infertility(Liu *et al.* , 2017*). MALAT1*, also known as nuclear-enriched abundant transcript 2 (*NEAT2*), was first described to be associated with metastasis of lung cancer (Ji *et al.* , 2003). Subsequently, a large number of studies focused on *MALAT1* were carried out. *MALAT1* expression is enhanced in multiple cancerous tissues, and *MALAT1* is implicated in the proliferation, apoptosis, migration, invasion and metastatic spread of tumor cells (Gutschner *et al.* , 2013). Endometriosis can be regarded as a benign metastatic disease, and furthermore, due to the ability of endometrial tissue to invade, metastasize and recur like tumors, it is very similar to cancer (Johnson and Hummelshoj, 2013). Epidemiological data suggest that

153 endometriosis does have malignant potential (Nezhat *et al.* , 2008). Moreover, Liang
154 *et al*. (Liang *et al.* , 2017) found that the expression of the lncRNA *MALAT1* was
155 significantly up-regulated in ectopic endometrial tissues compared with eutopic
156 endometrial tissues. This study is the first to report an association between
157 endometriosis and *MALAT1*, though this association was not found in other functional
158 and mechanistic studies on the role of *MALAT1* in reproductive medicine.

159 In this study, we first assessed *MALAT1* expression levels after controlled ovarian
160 stimulation (COS) in mural GCs from pre-ovulatory follicles from endometriosis
161 patients and from control patients. Then, we explored the potential role of *MALAT1*
162 in GC proliferation to provide new insights into the pathogenesis of
163 endometriosis-related infertility. In consideration of the difficulties in obtaining
164 human GCs in sizable amounts and maintaining primary cultures, we used the ovarian
165 granulosa-like tumor cell line, KGN, for further functional and mechanistic studies.
166 This cell line is considered to be an extremely useful model for understanding the
167 regulation of cell proliferation, apoptosis and steroidogenesis in human GCs (Nishi *et*
168 *al.* , 2001).

169

170 # Materials and Methods

171 This study was approved by the Ethics Committee of Nanfang Hospital of Southern
172 Medical University. Written informed consent was obtained from all patients.

173 **Patient samples and inclusion criteria**

174 GCs were collected from patients with and without endometriosis who were

undergoing IVF or ICSI treatment at the Center for Reproductive Medicine, Department of Gynecology and Obstetrics in Nanfang Hospital, People's Republic of China, in the period from March 2014 to December 2017. All endometriosis patients were diagnosed by a diagnostic and/or therapeutic laparoscopy or laparotomy, and uterine adenomyosis and malignant neoplasms were excluded. Staging of endometriosis was based on the revised American Society for Reproductive Medicine (rASRM) criteria. The inclusion criteria for control women were as follows: basal FSH < 10 IU/ml; basal estradiol (E_2) < 50 pg/ml; antral follicle count (AFC) > 5; regular menstrual cycles occurring every 25–35 days; these women entered the IVF/ICSI programme with non-endometriosis indications, and they were limited to male factor and tubal disease. The COS protocol for patients undergoing IVF/ICSI treatment consisted of recombinant FSH (Gonal F, Merck Serono, Modugno, Italy), highly purified FSH (Lishenbao, Livzon, Guangdong, China) and cetrorelix (Cetrotide, Merck Serono, Halle, Germany) stimulation followed by triptorelin (Diphereline, IPSEN, Signes, France) and hCG (Libao Biochemistry Co. Zhuhai, China) administration 34 to 36 h before oocyte retrieval. The basal serum sex hormones of patients on day 2–3 of the menstrual cycle was measured using a chemiluminescence kit (Roche).

GC collection

GC samples were purified as previously described (Kaur *et al.* , 2012). Follicular fluid was sampled by transvaginal ultrasound-guided puncture and follicles ≥ 10 mm in diameter were aspirated. The aspirates were centrifuged at 400×g for 10 min to

separate the fluid from the cells. Then the cell pellets were resuspended in media, layered over a 50% Percoll:PBS (Percoll; GE Healthcare, Uppsala, Sweden) solution, and centrifuged at 400×g for 20 min to remove red blood cells. The GCs at the interface were collected and contaminating erythrocytes were completely removed by erythrocyte lysis buffer (Sigma), whereupon the GCs were washed again with PBS. The final cell pellet was used for RNA analysis.

Cell line and culture

The human GC tumor-derived cell line, KGN, was a gift from Professor Ying-ying Qin at Shandong University, Shandong, People's Republic of China. Cells were maintained at 37°C in a humidified incubator containing 5% CO_2 in DMEM/nutrient mixture F-12 Ham (DMEM/F-12, HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA).

Cell transfection

The following locked nucleic acid (LNA) GapmeRs (Exiqon, Vedbaek, Denmark) were used to target *MALAT1*: *MALAT1* GapmeR-1: 628764 (batch number), *MALAT1* GapmeR-2: 5'-AGATTCCGTAACTTTA-3'. The sequence of the control LNA GapmeR (GapmeR Ctrl) was 5'-AACACGTCTATACGC-3'. KGN cells were transfected at 30%–40% confluency with 20 nM LNA GapmeRs targeting *MALAT1* or GapmeR Ctrl using Lipofectamine RNAiMax (Life Technologies, Carlsbad, CA, USA) in accordance with manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was used to examine the efficiencies of lncRNA knockdown.

RNA isolation and qRT-PCR

Total RNA from cultured cells and GCs was isolated using RNAiso Plus (TaKaRa, Dalian, China) in accordance with the manufacturer's protocol. Nuclear and cytoplasmic extracts were prepared in accordance with the instructions of the Nuclear/Cytoplasmic Isolation Kit (PARIS Kit, Life Technologies). For measuring mRNAs or lncRNAs, RNA (1 μg) was then reverse transcribed using a PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa) in a 20-μl reaction. cDNA was used as template for qRT-PCR using a SYBR Green PCR kit (TaKaRa) and LightCycler 480 Software (Roche). GAPDH was used as an internal control for quantification of target genes. Analysis of relative RNA expression levels was performed using the formula $2^{-\Delta\Delta CT}$. Sequences of primers used to amplify *MALAT1*, *GAPDH*, *U6* and pre-*GAPDH* are listed in Supplementary Table SI.

Cell counting kit-8 assays, ethynyl-2-deoxyuridine assays, flow cytometry and western blot analyses

Cell counting kit-8 (CCK-8) assays, ethynyl-2-deoxyuridine (EdU) assays, flow cytometry and western blot analyses were carried out as previously described (Liu *et al.*, 2017). More details can be found in the Supplementary Data.

Inhibition of the ERK/MAPK pathway by U0126

KGN cells were seeded onto a six-well plate and incubated at 37°C overnight. The cells were treated with 20 μM U0126 (Beyotime, China), a mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) inhibitor that inhibits MEK1/2 for down-regulation of phosphorylated ERK1/2 (p-ERK1/2) for 24 h. Then, the cells were transfected with the corresponding LNA GapmeR for 48 h.

Statistical analyses

Data were analyzed with SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) using either a Student's *t*-test for quantitative data with a Gaussian distribution or the Mann–Whitney U test for data with a non-Gaussian distribution. One-way ANOVA was used to analyze the differences between groups. The least squares difference method of multiple comparisons was used when the ANOVA results were statistically significant. The Kruskal–Wallis test was used for comparing two or more independent samples of equal or different sample size. The results obtained are expressed as the mean ± SD from at least three independent experiments. Correlations between *MALAT1* and clinical indices of patients were analyzed by Pearson's rank correlation. A P-value of $P < 0.05$ was considered statistically significant.

Results

Expression of *MALAT1* in endometriosis GCs

We analyzed *MALAT1* expression by qRT-PCR in a total of 104 GC samples, from 52 endometriosis patients and 52 matched controls. *MALAT1* was significantly down-regulated in endometriosis GCs as compared with controls ($P < 0.001$; Fig. 1A). Next, we compared the expression levels of *MALAT1* between 21 patients with ovarian endometriotic lesions and 31 patients with peritoneal endometriotic lesions ($P = 0.258$), between 21 patients with ovarian endometriotic lesions and 52 controls ($P = 0.001$), and between 31 patients with peritoneal endometriotic lesions and 52 controls

(P = 0.002). The results indicate that the presence of ovarian lesions in endometriosis patients had no effect on the expression of *MALAT1*. Furthermore, we found a significantly decreased *MALAT1* expression levels in women diagnosed with moderate and severe endometriosis (n = 16; Stage III-IV) following the rASRM classification compared with patients with surgically confirmed minimal and mild endometriosis (n = 36; Stage I-II) (P = 0.01; Fig. 1B). The characteristics of the endometriosis patients and controls are shown in Table I, which indicates that endometriosis has a negative impact on the outcome of IVF in comparison to controls with tubal disease or male factor infertility, including a significantly lower AFC, fewer ≥ 14-mm follicles on hCG day, fewer oocytes obtained, fewer mature oocytes and fewer good quality embryos. Next, we analyzed the association between *MALAT1* and clinical features in the 104 patients mentioned above. As shown in Table II, statistical analyses indicated a weak negative correlation between *MALAT1* lncRNA expression and age, and positive correlations between *MALAT1* lncRNA expression and AFC, basal progesterone, normal fertilization, and the numbers of ≥ 14-mm follicles on hCG day, oocytes retrieved, mature oocytes, available embryos and good-quality embryos; no significant correlations were observed between *MALAT1* lncRNA expression levels and other clinical characteristics.

MALAT1 is a potential biomarker for endometriosis

To investigate the characteristics of *MALAT1* as a potential biomarker for endometriosis, ROC curves were drawn, and the areas under the ROC curves (AUCs) were calculated using data from different groups. *MALAT1* expression levels were

obtained from the qRT-PCR data from the cohort of 104 patients (36 Stage I-II versus 16 Stage III-IV versus 52 controls). The AUC was 0.705 (95% CI: 0.606–0.804; $P < 0.001$) for all endometriosis patients and controls, 0.651 (95% CI: 0.536–0.767; $P = 0.016$) for Stage I-II endometriosis and controls, and 0.827 (95% CI: 0.718–0.936; $P < 0.001$) for Stage III-IV endometriosis and controls, suggesting that *MALAT1* has potential diagnostic value in endometriosis; these results illustrate a strong separation between the Stage III-IV endometriosis patients and the control group (Figs 2A, B and C).

Distribution of *MALAT1* and the effect of *MALAT1* knockdown on GC proliferation

The relationship between *MALAT1* RNA distribution and other structural and functional entities will provide important insights into its function. We used qRT-PCR to analyze RNA from nuclear and cytoplasmic fractions in the KGN cell line. The results indicate that *MALAT1* was primarily distributed in the nucleus (Fig. 3A). We thus silenced *MALAT1* expression with LNA GapmeRs in a KGN cell line. As shown in Fig. 3B, LNA GapmeRs could effectively inhibit the expression of *MALAT1* ($P < 0.001$). To determine the effect of *MALAT1* knockdown on cell proliferation, we performed a CCK-8 assay and an EdU assay in KGN cells. Both of the results show that *MALAT1* knockdown remarkably attenuated cell viability (Figs 3C, D and E).

***MALAT1* knockdown induces G0/G1 cell cycle arrest by promoting P21 expression**

To elucidate the mechanism by which *MALAT1* affected cell proliferation, flow

cytometry analysis was performed to analyze differences in cell-cycle distributions after *MALAT1* knockdown for 48 h. The results show that *MALAT1* knockdown increased the percentage of cells in the G0/G1 phase and decreased the percentage of cells in the S and G2/M phases, as compared with the control group (Figs 4A and B). The orderly progression of the cell cycle is orchestrated by cyclin dependent kinases (CDKs), which are activated by binding to cyclins. P21, encoded by *CDKN1A*, which is the first identified member of the cyclin-dependent kinase inhibitors (CKIs). P21 can inhibit the activity of each member of the cyclin/CDK family, and overexpression of P21 inhibits the proliferation of mammalian cells (Xiong *et al.* , 1993). P21 is regarded as a major mediator of G1 growth arrest (Sherr and Roberts, 1995). As *MALAT1* does not directly inhibit cell proliferation, we attempted to identify its target genes. *MALAT1* has been shown to regulate the cell cycle by repressing the expression of P21 (Wang *et al.* , 2016). Accordingly, we analyzed *P21* expression levels after *MALAT1* knockdown. As expected, the mRNA levels of *P21* were significantly increased by *MALAT1* GapmeR-1 ($P = 0.008$; Fig. 4C) and *MALAT1* GapmeR-2 ($P = 0.031$; Fig. 4C). Then, we investigated whether the expression of the *P21* was affected by *MALAT1* in the same cohort of patients, and we found that the expression of *P21* was significantly elevated in endometriosis GCs compared with controls ($P < 0.001$; Fig. 4D). Further analysis of the relationship between *MALAT1* and *P21* in the 104 GC samples of patients revealed a negative correlation ($R= -0.628$; $P < 0.001$; Fig. 3E). In addition, higher protein levels of P21 and P53 and lower protein levels of CDK2 and cyclin D1 in *MALAT1* knockdown cells were observed

(Figs 4F and G). P53 is widely recognized as a protein functioning during the cell cycle. Activated P53 causes G1 arrest by inducing P21, followed by an inhibition of cyclin/CDK (Vermeulen *et al.* , 2003). Cyclin D and CDK2 are key regulators that are required for the G1/S phase (Neganova *et al.* , 2011). In conclusion, *MALAT1* knockdown led to an increase in *P21* mRNA levels and to an increase in P53 protein levels, followed by an inhibition of cyclin D1/CDK2, which controlled the G0/G1 cell-cycle arrest.

In addition, we further analyzed the associations between *P21* and clinical features in the 104 patients mention above. As shown in Table III, statistical analyses indicated negative correlations between *P21* expression and AFC ($R= -0.509$, $P <0.001$), the number of ≥ 14-mm follicles on hCG day ($R = -0.233$, $P = 0.017$) and follicles aspirated ($R = -0.331$, $P = 0.001$), oocytes retrieved ($R = -0.265$; $P = 0.007$), mature oocytes ($R = -0.266$, $P = 0.008$) and good-quality embryos ($R = -0.250$, $P = 0.013$), and a positive correlation between *P21* expression and age ($R = 0.276$; $P = 0.005$).

Effects of *MALAT1* knockdown on MAPK and PI3K/AKT pathways

The MAPK pathway, mainly including ERKs, c-Jun N-terminal protein kinases (JNKs) and P38 MAPK subfamilies, and the phosphatidylinositol 3-kinase (PI3K)/AKT pathways have been shown to be crucial and are intensively explored intracellular signaling pathways in *MALAT1*-induced cell proliferation (Chen *et al.* , 2016, Dong *et al.* , 2015, Zhao *et al.* , 2015). Therefore, we focused on these two signaling pathways and performed western blot analysis to investigate alterations in the activities of these pathways upon *MALAT1* knockdown in KGN cells. As shown in Fig. 5, *MALAT1*

knockdown significantly increased the levels of phosphorylated ERK1/2 (p-ERK1/2) and p-JNK, and decreased the levels of p-P38 MAPK. Therefore, our results suggest that *MALAT1* knockdown led to the activation of the ERK/JNK pathways and inactivation of P38 MAPK pathway, while we found no significant differences in the PI3K/AKT pathway.

MALAT1 regulates P21 expression in an ERK/MAPK pathway-dependent manner

Many studies indicated that prolonged activation of the MAPK pathway is associated with a reduction in CDK activity, mediated by increased expression of P21 (Adorisio *et al.* , 2018, Park *et al.* , 2000, Tombes *et al.* , 1998). Therefore, in order to prove the conjecture that the activation of the ERK/MAPK pathway caused by *MALAT1* knockdown could promote the activation of P21 and its upstream target P53, the addition of U0126, a ERK/MAPK kinase inhibitor that can completely block the phosphorylation of ERK, was used to pre-treat KGN cells. Our results show that in the presence of U0126, the up-regulation of p-ERK1/2, P21 and P53 and the down-regulation of cyclin D1 and CDK2 by the knockdown of *MALAT1* were attenuated (Fig. 6). These data indicate that the activation of the ERK/MAPK pathway by *MALAT1* knockdown promoted the activation of the P21/P53 pathway and further caused CDK2 and cyclin D1 inactivation.

Discussion

In this study, we investigated the role of *MALAT1* lncRNA in endometriosis and its

associated infertility. We first found that *MALAT1* was down-regulated in endometriosis GCs and was associated with the AFC. The expression levels of *MALAT1* lncRNA were significantly lower in the GCs of infertile women with advanced stages of endometriosis. *MALAT1* has potential diagnostic value in endometriosis, as its expression levels showed a significant difference between Stage III-IV endometriosis patients and the control group. *MALAT1* was primarily localized in the nuclei of GCs. We then provided evidence that *MALAT1* knockdown inhibited GC proliferation by restraining the cell-cycle in the G0/G1 phase. We found a negative correlation between *MALAT1* lncRNA and *P21* mRNA in KGN cells as well as in primary cells from patients. The increase in P21 and P53 protein levels by *MALAT1* knockdown was probably caused by activation of the ERK/MAPK pathway.

Endometriosis is a complex disease affecting women of reproductive age, and it can cause infertility. Although the cause of endometriosis-associated infertility remains elusive, genetic abnormalities are believed to contribute to this process. Recent studies revealed that the sequences of lncRNAs cover a larger fraction of the human genome than do protein-coding genes. LncRNAs were initially considered to be spurious transcriptional noise; however, they have recently emerged as key players in fundamental cellular processes and diseases (Mercer *et al.* , 2009, Wilusz *et al.* , 2009). Highly conserved sequences are commonly known to exert important physiological functions, and lncRNAs are no exception. The four dimensions of lncRNA conservation include the sequence, structure, function and expression from syntenic loci (Diederichs, 2014). *H19* lncRNA, a highly abundant and conserved

imprinted gene, represents the first example of an lncRNA involved in endometriosis and its associated infertility (Ghazal *et al.*, 2015), and it has been implicated in many essential biological processes and diseases.

MALAT1 also stands out from the lncRNA family due to its high evolutionary conservation and abundant expression amongst mammals. *MALAT1* was first demonstrated to be associated with non-small cell lung cancer (Ji *et al.*, 2003). Subsequently, *MALAT1* was identified in multiple types of physiological processes, including alternative splicing, nuclear organization and epigenetic modulating of gene expression. *MALAT1* is found distributed in the nucleus, where it localizes to nuclear speckles and paraspeckles (Clemson *et al.* , 2009, Quinn and Chang, 2016), which is consistent with our finding in the KGN cell line. Nuclear speckles are dynamic subnuclear structures containing pre-messenger RNA splicing factors and other proteins involved in transcription, 3'-end RNA-processing and reversible protein phosphorylation (Lamond and Spector, 2003).

A growing number of studies have proved that *MALAT1* plays an important role in the proliferation and metastasis of cancers. However, little is known about the role of *MALAT1* in endometriosis and its associated infertility. Therefore, our study is the first to measure the expression levels of *MALAT1* in endometriosis GCs, and we found that *MALAT1* was obviously down-regulated in endometriosis GCs and its expression levels were weakly positively correlated with AFC ($R = 0.376$; $P < 0.001$). The AFC test is considered the preferred method for predicting the ovarian reserve (Hendriks *et al.* , 2005). However, it is not known whether a reduced AFC or a

diminished ovarian reserve in endometriosis women is associated with the decrease in *MALAT1* expression levels in GCs, as substantial data to support this hypothesis are still warranted. In addition, *MALAT1* levels were significantly lower in women with Stage III-IV endometriosis compared with women with Stage I-II endometriosis, suggesting that *MALAT1* levels are related to the severity of endometriosis.

Even today the gold standard for the diagnosis of endometriosis remains direct visualization of lesions, preferably coupled with histologic confirmation of the presence of endometrial glands and stroma in biopsies of suspected lesions. The enigmatic pathophysiology of endometriosis presents unique challenges to biomarker development that are now well outlined. Nevertheless, with the progress of RNA sequencing technology, promising biomarker candidates are emerging, many of which are ncRNAs. Reduced plasma levels of *miR-17-5p*, *miR-20a* and *miR-22* (Suryawanshi *et al.* , 2013) and elevated plasma levels of *miR-16*, *miR-191* and *miR-195* (Wang *et al.* , 2013) have been identified as biomarkers to discriminate between patients with and without endometriosis. In our study, we have drawn ROC curves to demonstrate that *MALAT1* has potential diagnostic value in endometriosis, though further study and more substantial data will certainly be needed.

Based on the down-regulation of *MALAT1* in endometriosis GCs and its intranuclear distribution, we used LNA GapmeRs to knock down *MALAT1* in KGN cells to examine the impact of *MALAT1* knockdown. The results show that knockdown of *MALAT1* led to decreased cell viability and restrained cell-cycle progression. Evidence has shown that the local intrafollicular environment of

439 endometriosis women is characterized by alterations in the function of the GCs
440 (Sanchez *et al.* , 2016). Taken together, the down-regulation of *MALAT1* in
441 endometriosis GCs may have a negative impact on the growth and development of
442 oocytes by inhibiting GC proliferation and cell cycle progression, leading to an
443 inferior IVF outcome in women with endometriosis, including a lower AFC, fewer
444 ≥ 14-mm follicles on hCG day, fewer oocytes obtained, fewer mature oocytes, and
445 fewer good quality embryos.

446 LncRNAs attenuate cell proliferation through diverse mechanisms, including the
447 inhibition of cell-cycle progression and the promotion of apoptosis. According to our
448 results, *MALAT1* attenuated cell proliferation by inhibiting cell-cycle progression.
449 Cell-cycle progression is mediated by the sequential activation of members of the
450 CDK families; a CDK binds a regulatory cyclin protein, and most cyclins promote
451 CDK activity, whereas CKIs inhibit CDK activity. P21 is the first identified member
452 of the CKIs. We thus investigated the association between *MALAT1* and *P21*, and we
453 found a negative correlation between them both in the KGN cell line and in the GCs
454 from patients.

455 Regulation of P21 expression is complex. Transcription of the *P21* gene involves
456 P53-dependent and -independent mechanisms, while protein levels are controlled in
457 part by proteasome-mediated degradation (Cazzalini *et al.* , 2010, Olszewska *et al.* ,
458 2013). P53 is widely recognized as a protein functioning during the cell cycle, and
459 activated P53 can cause a G1 arrest by activating P21. CDK2 has been implicated in
460 the control of the G1 to S phase transition, and it was associated with cyclin A, D, and

E. Harper et al. (Harper *et al.* , 1993) identified P21 as a CDK2-interacting protein in a yeast two-hybrid screen. Consequently, combined with the flow cytometry analysis results, we can draw the conclusion that *MALAT1* knockdown arrested the cell-cycle in the G0/G1 phase by increasing P21 expression in a P53-dependent manner.

In view of the knowledge that the PI3K/AKT and MAPK pathways are known to be associated with *MALAT1*-related cell proliferation (Chen *et al.*, 2016, Dong *et al.*, 2015, Zhao *et al.*, 2015), we investigated the effects of *MALAT1* knockdown on these two pathways in KGN cells. Our results show that the ERK/MAPK pathway was aberrantly activated in *MALAT1*-silenced cells, and increased ERK1/2 phosphorylation by *MALAT1* knockdown was suppressed by the addition of U0126, further suggesting that *MALAT1* was involved in GC proliferation via the ERK/MAPK pathway. The PI3K/AKT pathway may have no influence on *MALAT1*-induced GC proliferation. Moreover, we found that inhibition of ERK1/2 phosphorylation by U0126 could decrease the high levels of P21 and P53 protein caused by MALAT1 knockdown, suggesting that the activation of the ERK/MAPK pathway could stabilize the P21 protein with a concomitant increase in P53 protein, which is consistent with other previous studies (Adorisio *et al.*, 2018, Park *et al.*, 2000, Tombes *et al.*, 1998). The activation of the P21/P53 pathway further caused CDK2 and cyclin D1 inactivation, leading to an arrest of the cell cycle in the G0/G1 phase.

The MAPK pathway is a well-known transducer of signals that regulate proliferation, and the ERK pathway is definitely the best-characterized MAPK

pathway. P38 inhibits ERK signaling directly or by regulating the activity of protein phosphatase 2A (PP2A), which dephosphorylates MEK1/2 (Hutchison, 2012). Accordingly, ERK activation is followed by a reduction in P38 phosphorylation. However, the mechanism whereby the ERK pathway suppresses P38 activation is unknown. Moreover, a previous study suggested that JNK can also inhibit P38 (Peng *et al.* , 2009). Precise patterns of activation of the ERK, JNK and P38 pathways and interactions between them are crucial to a wide variety of proliferation programmes, but how these pathways interact in different tissues varies.

The results of this study are a little different from others. The ERK/MAPK pathway is often aberrantly activated in human cancers and stimulates cell proliferation. Interestingly, in our study, the ERK/MAPK pathway was activated after *MALAT1* knockdown in KGN cells, and the activation of the ERK/MAPK pathway contributed to the suppression of cell proliferation, as also described in a previous study on glioma cells (Han *et al.* , 2016). Therefore, the direct link between the ERK/MAPK pathway and proliferation remains unclear and requires further study. Besides, a few limitations exist in this study. Firstly, the hormonal treatment used in IVF and surgical removal of endometriotic lesions may alter *MALAT1* expression in GCs. Moreover, because of the multifaceted nature of endometriosis, a single genetic signal is not sufficient to account for the considerable genetic susceptibility for this disease. Thus, future studies targeted at the interaction of the genetic network, including DNA, RNA and proteins, will be of great help.

In conclusion, women with endometriosis had decreased *MALAT1* expression

levels in GCs, and the expression of *MALAT1* was associated with the AFC as well as the severity of endometriosis. *MALAT1* has potential diagnostic value in endometriosis. Knockdown of *MALAT1* in KGN cells obviously inhibited cell proliferation, caused by P21/P53-mediated cell-cycle arrest, and the activation of the ERK/MAPK pathway participated in this process. Therefore, our study suggests that altered *MALAT1* expression levels in GCs in women with endometriosis may impair fertility, providing new insights into the pathogenesis of endometriosis and its associated infertility.

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Authors' roles

The specific work of each author in this study was as follows. Substantial contributions to conception and design, acquisition of data or analysis and interpretation of data: Ying Li, Yu-dong Liu, Shi-ling Chen, Xin Chen, De-sheng Ye, Xing-yu Zhou, Jing Zhe and Jun Zhang. Writing the first draft and revising it critically for important intellectual content: Ying Li, Yu-dong Liu and Shi-ling Chen. Final approval of the version to be published: Ying Li, Yu-dong Liu, Shi-ling Chen, Xin Chen, De-sheng Ye, Xing-yu Zhou, Jing Zhe and Jun Zhang.

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Conflict of interest

The authors have no conflict of interest to declare.

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Figure 1 Expression of (*MALAT1*) in endometriosis granulosa cells.

(A) The expression levels of lung adenocarcinoma transcript 1 (*MALAT1)* in endometriosis granulosa cells (GCs) (n = 52) were lower than in non-endometriosis GCs (n = 52, ***P < 0.001). (B) *MALAT1* levels were significantly lower in women with Stage III-IV endometriosis (n = 16) compared with women with Stage I-II endometriosis (n = 36, *P = 0.01).

Figure 2 The ROC curve of *MALAT1* expression levels in GCs for distinguishing endometriosis from normal controls.

(A) all endometriosis patients (n = 52). (B) Stage I-II endometriosis patients (n = 36).

(C) Stage III-IV endometriosis patients (n = 16).

Figure 3 Distribution of *MALAT1* and the effect of *MALAT1* knockdown on cell proliferation.

(A) Fractionation of KGN cells followed by quantitative RT-PCR (qRT-PCR). The efficiency of the fractionation was assessed by quantifying cytoplasmic mRNA levels of *GAPDH*, and nuclear RNA levels of pre-*GAPDH* and U6 small nuclear RNA. (B) The transfection efficiency was determined 48 h after incubation with 20 nM locked nucleic acid (LNA) GapmeRs targeting *MALAT1* or GapmeR control (GapmeR Ctrl), and the relative *MALAT1* expression levels were measured by qRT-PCR. (C) Cellular proliferation of untransfected or transfected KGN cells was measured using cell counting kit-8 assays for 24–96 h. (D and E) In an ethynyl-2-deoxyuridine incorporation assay, the percentage of proliferating cells (red/DAPI) differed between cells with LNA GapmeRs targeting *MALAT1* and cells with GapmeR Ctrl (200× magnification). Results are expressed as the mean ± SD from at least three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 by a two-tailed Student's *t*-test.

Figure 4 Effect of *MALAT1* knockdown on the cell cycle and P21 expression.

(A and B) Flow cytometry analysis showed a significant increase in cells in the G0/G1 phase and a significant decrease in cells in the S and G2/M phases in *MALAT1*-silenced KGN cells (48 h post-transfection). (C) The cyclin dependent

kinase inhibitor 1A, *CDKN1A* (*P21)* mRNA levels were significantly increased in *MALAT1* GapmeR-1 (P = 0.008) and *MALAT1* GapmeR-2 (P = 0.031) cells. (D and E) Expression of *P21* was significantly elevated in endometriosis GCs compared with controls (P < 0.001), and negatively correlated with *MALAT1* in the 104 GC samples of patients (R = −0.628; P < 0.001). (F and G) Western blot analysis showed that *MALAT1* knockdown increased the levels of P21 and P53 and decreased the levels of cyclin D1 and CDK2.

Figure 5 Alternations in the activities of MAPK and the phosphatidylinositol 3-kinase/AKT pathways upon *MALAT1* knockdown in KGN cells.
(A and B) Phosphorylated and total mitogen-activated protein kinase (MAPK), mainly including extracellular signal-regulated kinases (ERKs), c-Jun N-terminal protein kinases (JNKs) and P38 MAPK subfamilies, were detected by western blot analysis. The results show that *MALAT1* knockdown significantly increased the levels of phosphorylated ERK1/2 (p-ERK1/2) and p-JNK, and decreased the levels of p-P38 MAPK. (C and D) Phosphorylated and total phosphatidylinositol 3-kinase (PI3K) and AKT levels were measured by western blot analysis. No significant differences were observed in the PI3K/AKT pathway.

Figure 6 Effect of the ERK/MAPK inhibitor (U0126) on *MALAT1* knockdown-induced alteration of p-ERK, P21, P53, CDK2 and cyclin D1 levels. U0126 (20 μM) was added to the 1% FBS medium for 24 h as indicated. Then, the

cells were transfected with the corresponding LNA GapmeRs for 48 h. Phosphorylated and total levels of ERK1/2, P21, P53, CDK2 and cyclin D1 were measured by western blot analysis. The results show that in the presence of U0126, the up-regulation of p-ERK1/2, P21 and P53 and the down-regulation of cyclin D1 and CDK2 by the knockdown of *MALAT1* were attenuated.

Table I Characteristics of the endometriosis and control patients whose samples were used for qRT-PCR. analyses.

Characteristics		Control (n = 52)	EM (n = 52)	*P*-value
Age (years)		31.94 ± 3.55	32.31 ± 4.36	0.640
Types of infertility	Primary	61.54% (32/52)	42.31% (22/52)	0.038*
	Secondary	38.46% (20/52)	57.69% (30/52)	
Infertility years		4.14 ± 2.57	4.75 ± 3.40	0.396
BMI (kg/m^2)		21.00 ± 2.06	20.88 ± 2.35	0.775
E_2 (pg/mL)		45.96 ± 27.95	50.92 ± 29.22	0.386
TT (ng/mL)		0.35 ± 0.47	0.30 ± 0.29	0.576
PRL (ng/mL)		18.81 ± 9.13	18.20 ± 8.25	0.319
FSH (mIU/mL)		6.81 ± 2.06	7.63 ± 2.55	0.077
LH (mIU/mL)		4.48 ± 1.45	5.08 ± 1.69	0.058
P4 (ng/mL)		0.62 ± 0.53	0.60 ± 0.57	0.858
AFC		13.83 ± 3.74	10.25 ± 5.01	0.000***
Starting Gn dose (IU)		211.78 ± 64.62	235.29 ± 66.41	0.072
Total Gn dose (IU)		2202.92 ± 706.11	2597.50 ± 840.66	0.012*
Total Gn days		9.69 ± 1.78	10.04 ± 1.77	0.322
Number of ≥ 14-mm follicles on hCG day		9.32 ± 3.41	7.41 ± 3.52	0.005**
E_2 in trigger day (pg/mL)		2174.48 ± 924.75	2453.56 ± 1466.70	0.254

No. of follicles aspirated	15.39 ± 4.64	12.25 ± 6.74	0.007**
No. of oocytes retrieved	11.41 ± 3.24	8.67 ± 5.39	0.003**
No. of mature oocytes	9.96 ± 3.23	7.45 ± 4.60	0.002**
No. of available embryos	4.22 ± 2.86	3.38 ± 2.71	0.135
No. of good-quality embryos	3.10 ± 2.47	2.00 ± 2.02	0.017*

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by a Student's *t*-test for quantitative data with a Gaussian distribution or the Mann-Whitney U test for data with a non-Gaussian distribution. Data are mean ± SD. qRT-PCT: quantitative real-time PCR; EM: endometriosis; E2: estradiol; TT: total testosterone; P4: progesterone; AFC: antral follicle count; Gn: gonadotrophin.

Table II Pearson's rank correlation coefficients of the expression of *MALAT1* lncRNA and patients' characteristics.

	R	*P*-value
Age (years)	−0.290	0.003**
BMI (kg/m^2)	−0.047	0.636
E_2 (pg/mL)	−0.050	0.621
TT (ng/mL)	0.055	0.589
FSH (mIU/mL)	−0.182	0.066
LH (mIU/mL)	−0.148	0.137
P4 (ng/mL)	0.224	0.024*
PRL (ng/mL)	0.046	0.652
AFC	0.376	0.000***
Number of ≥ 14-mm follicles on hCG day	0.316	0.001**
No. of follicles aspirated	0.306	0.002**
No. of oocytes retrieved	0.267	0.007**
No. of mature oocytes	0.309	0.002**
Normal fertilization	0.203	0.039*
No. of available embryos	0.267	0.008**
No. of good-quality embryos	0.285	0.004**

n = 104; *$P < 0.05$, **$P < 0.01$, ***P < 0.001.

MALAT1: lung adenocarcinoma transcript 1; lncRNA: long non-coding RNA.

Table III Pearson's rank correlation coefficients of the expression of *P21* mRNA and patients' characteristics.

	R	*P*-value
Age (years)	0.276	0.005**
BMI (kg/m^2)	−0.014	0.884
E_2 (pg/mL)	−0.023	0.823
TT (ng/mL)	0.097	0.340
FSH (mIU/mL)	0.188	0.057
LH (mIU/mL)	0.029	0.770
P4 (ng/mL)	0.115	0.250
PRL (ng/mL)	−0.095	0.349
AFC	−0.509	0.000***
Number of ≥ 14-mm follicles on hCG day	−0.233	0.017*
No. of follicles aspirated	−0.331	0.001**
No. of oocytes retrieved	−0.265	0.007**
No. of mature oocytes	−0.266	0.008**
Normal fertilization	−0.152	0.124
No. of available embryos	−0.179	0.070
No. of good-quality embryos	−0.250	0.013*

n=104; *P < 0.05 , **P < 0.01, ***P < 0.001.