Abstract. It has been reported that long non-coding RNA nuclear-enriched abundant transcript 1 (NEAT1) is involved in follicular growth and multiple ovarian diseases, but not the physiological function of NEAT1 in mouse granulosa cells (mGCs). Therefore, the aim of the present study was to investigate the biological roles and regulatory mechanisms of NEAT1 in mGCs. The biological effects of NEAT1 on mGCs proliferation, apoptosis, production of 17β-Estradiol (E2) and progesterone (P4) were investigated using MTS, flow cytometry and enzyme-linked immunosorbent assays, respectively. The association between NEAT1 and microRNA (miR)-874-3p was verified using luciferase reporter assay and RNA immunoprecipitation analysis. The results demonstrated that the knockdown of NEAT1 in mGC cells significantly promoted mGCs cell proliferation, inhibited apoptosis and increased the production of E2 and P4 in mGCs. The interference-mediated effect of NEAT1 on mGCs could be partially reversed by the downregulation of miR-874-3p. Overall, these results indicated that NEAT1 served as a competing endogenous RNA by competitively binding with miR-874-3p, thereby modulating mGCs proliferation and the production of E2 and P4 in mGCs.

Introduction
Follicular atresia is an inevitable degenerative process that occurs at all stages of follicular development in mammals (1,2). Among domesticated animals, sheep and cows, >99% of these follicles undergo atresia (3-5), however the regulatory mechanism of follicular atresia remains largely unclear. Granulosa cells (GCs) are shown to play a key role in the processes of follicular development and atresia (5). GC proliferation and estradiol synthesis is closely associated with follicular atresia (6,7). Thus, elucidating regulatory mechanisms such as follicular GC proliferation and estradiol synthesis is important to decrease the occurrence of these atresia processes.

The growth, differentiation and apoptosis of GCs are modulated by a large number of molecules including non-coding RNAs (ncRNAs) genes (8,9). Among ncRNAs, long ncRNAs (lncRNAs), which are defined as transcripts >200 nucleotides without protein-coding ability, have gained widespread attention in disease diagnosis and treatment (10,11). In the field of reproduction, dysregulation of lncRNAs has been shown to play important roles in ovarian function, follicle development and luteal formation in humans, mice, bovines and pigs (12,13). LncRNA nuclear-enriched abundant transcript 1 (NEAT1) has been reported to be highly expressed in multiple types of cancer, such as breast, colorectal and thyroid cancer, and plays an oncogenic role in tumorigenesis by regulating cell proliferation, apoptosis, invasion and metastasis (14,15). NEAT1 has been reported to be involved in polycystic ovary syndrome by regulating GC cell proliferation and apoptosis via the microRNA (miR)-381/insulin-like growth factor 1 (IGF1) axis (16). However, its functional roles and regulatory mechanisms in GC remain elusive. Therefore, the present study aimed to investigate the biological effects of NEAT1 on mouse GC proliferation, apoptosis and estradiol synthesis. Additionally, the regulatory mechanisms of NEAT1 in GCs were further explored through a series of experiments.

Materials and methods
Animals and follicles isolation. A total of 10 female C57BL/6j mice (age, 4-5 weeks; weight, 18-25 g) were obtained from
the Changchun Institute of Biological Products Co., Ltd, and housed at 21-25°C, humidity 52-60% and a 12-h light/dark cycle under specific pathogen-free conditions. All of the mice were provided a standard diet and sterile water ad libitum for one week. Animal care and experiments were done in accordance with the Principles for the Utilization and Care of Vertebrate Animals of the National Institutes of Health (17).

The behavior and health status of the mice were observed every day, and the mice were weighed every 2 days. The mice were sacrificed by inhalation of CO2 (50% of the chamber volume/min) before dissection for excision of ovarian tissue. Confirmation of mouse mortality was verified lack of heartbeat and dilation of pupils. Follicles were isolated from ovarian tissue according to a previous study (18). Follicles were divided into healthy follicles (HFs), early atretic follicles (EAFs) and progressively atretic follicles (PAFs) according to follicle morphological characteristics as previously described by Miller et al (19).

**Mouse granulosa cells (mGCs) isolation and culture.** Primary mGCs were isolated from follicles and cultured as described previously (20). Dulbecco's Modified Eagle medium/nutrient mixture F-12 (DMEM/F12; MilliporeSigma) was changed every 48 h. The two or three passage mGCs were selected for subsequent experiments. All experiments using laboratory animals were approved by the Animal Ethics Committee of Jilin Academy of Agricultural Sciences (approval no. JNK20210719-2; Changchun, China).

**mGCs transfection.** A siRNA that directly targeted mouse NEAT1 (si-NEAT1; 5'-TGTTAATGGTGGAGGAAG-3') and appropriate non-targeting siRNA (si-NC; 5'-GGCUCGAACGUGUCAGU-3') were bought from Takara Biotechnology Co., Ltd. In addition, Shanghai GenePharma Co., Ltd. provided miR-874-3p mimics (miR-874-3p; 5'-UGA GCU GUA AUC AGG UCC CGU C-3') or miR-NC control plasmid, namely NEAT1-mutant (MUT-NEAT1). scrambled negative control (miR-NC; 5'-UUGUACUACACAAAGUACUG-3'), miR-874-3p inhibitor (anti-mir-874-3p; 5'-GAC GCGGGACC UGAAUACAGCUA-3') as well as its scrambled negative control (anti-miR-NC; 5'-CAGUACUUUUGUUGUA CAA-3'). mGCs (5x10⁴ cells/well) were cultured in six-well plates until they reached 80% confluence. After 4 h of cell starvation in serum-free medium, mGCs were treated with serum-free medium for 48 h. The production of 17β-Estradiol (E2) and progesterone (P4) were measured using enzyme-linked immunosorbent assay (ELISA) kits (cat nos. E03E0023 and E03P0200; BlueGene) based on the manufacturer's instructions, respectively. The minimum detectable concentrations were 5 pg/ml for E2 and 0.2 ng/ml for P4.

**Steroid hormone detection.** Transfected cells were grown in serum-free medium for 48 h. The production of 17β-Estradiol (E2) and progesterone (P4) were measured using enzyme-linked immunosorbent assay (ELISA) kits (cat nos. E03E0023 and E03P0200; BlueGene) based on the manufacturer's instructions, respectively. The minimum detectable concentrations were 5 pg/ml for E2 and 0.2 ng/ml for P4.

**Isolation of cytoplasmic and nuclear RNA.** The Cytoplasmic and Nuclear RNA Purification kit (Norgen Biotek Corp.,) was used to isolate cytoplasmic and nuclear RNA from mGCs following the manufacturer's instruction. The expression of NEAT1 in cytoplasmic and nuclear RNA of mGCs was measured using RT-qPCR as mentioned above.

**Luciferase reporter assay.** The ENCORI database (https://starbase.sysu.edu.cn/) was used to decode the miR-874-3p-NEAT1 binding interaction. The wild-type sequence NEAT1 (WT-NEAT1) containing a binding target site of miR-874-3p (5'-UGAGCGUAUAUCAGGCGAC-3') was synthesized and inserted into a dual-luciferase miRNA target expression vector (pmirGLO; Promega Corporation). In addition, another reporter plasmid was conducted by inserting the mutated target sequence of miR-874-3p (5'-UGAGCGUAUAUCAGGCGAC-3') into a pmirGLO vector to form a negative control plasmid, namely NEAT1-mutant (MUT-NEAT1). Subsequently, both reporter plasmids and miR-874-3p mimics (5'-UGAGCGUAUAUCAGGCGAC-3') or miR-NC mimics (5'-UUGUACUACACAAGUGAU-3') were co-transfected into mGCs cells using Lipofectamine® 3000 reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Luciferase activity assay was
were washed six times with 500 µl cold RIP Wash Buffer for 10 min at 4˚C and the supernatant was discarded. The beads-antibody complex in 900 µl RIP Immunoprecipitation Buffer was centrifuged at 3,000 x g (cat. no. CC8039-01; As One Shanghai Corporation) for 3 h at 4˚C. Immunoprecipitation tubes were centrifuged at 3,000 x g for 10 min at 4˚C and the supernatant was discarded. The beads were washed six times with 500 µl cold RIP Wash Buffer (860 µl RIP Wash Buffer, 35 µl 0.5 M EDTA and 5 µl protease inhibitor cocktail and 0.25 µl RNase inhibitor). Cells were gently rotated using a rotary mixer for 1 h at 4˚C. Subsequently, ~5 µg human anti-argonaute-2 (Ago2; cat. no. MABE56; MilliporeSigma) or normal mouse immunoglobulin G (IgG; cat. no. 12-370; MilliporeSigma) was added to each tube and vortexed briefly. Subsequently, 100 µl of the supernatant was removed and added to each sample was normalized to Renilla luciferase activity.

RNA immunoprecipitation (RIP). The EZ-Magna RIP RNA-binding protein immunoprecipitation kit (cat. no. 17-701; Merck KGaA) was applied to analyze the association between miR-874-3p and NEAT1 in mGCs according to the manufacturer's instructions. Briefly, following washing of the cells in the flasks or plates twice with 10 ml ice-cold PBS, 2.0x10^7 mGCs were lysed using 100 µl RIP lysis buffer (cat no. CS203176, MilliporeSigma) containing 0.5 µl protease inhibitor cocktail and 0.25 µl RNase inhibitor. Cells were collected by centrifugation at 1,000 x g for 5 min at 4˚C and the supernatant was discarded, then 100 µl RIP lysis buffer was added for mGCs RIP lysis. Magnetic beads (protein A/G; cat no. CS203178; MilliporeSigma) were completely dispersed and re-suspended by pipetting according to the manufacturer's protocol, then 50 µl magnetic bead suspension was transferred to each tube, and 0.5 ml RIP Wash Buffer (cat. no. CS203177; MilliporeSigma) was added to each tube and vortexed briefly. Subsequently, ~5 µg human anti-argonaute-2 (Ago2; cat. no. MABE56; MilliporeSigma) or normal mouse immunoglobulin G (IgG; cat. no. 12-370; MilliporeSigma) as control antibodies was added for 4 h at 4˚C according to the manufacturer's protocol. A total of 100 µl mGCs RIP lysate was centrifuged at 3,000 x g for 10 min at 4˚C. The beads-antibody complex in 900 µl RIP Immunoprecipitation Buffer (860 µl RIP Wash Buffer, 35 µl 0.5 M EDTA and 5 µl RNase Inhibitor), and gently rotated using a rotary mixer (cat. no. CC8039-01; As One Shanghai Corporation) for 3 h at 4˚C. Immunoprecipitation tubes were centrifuged at 3,000 x g for 10 min at 4˚C and the supernatant was discarded. The beads were washed six times with 500 µl cold RIP Wash Buffer (cat. no. CS203177; MilliporeSigma). Ago2 or IgG complex (100 µl) isolation from beads was performed using 150 µl proteinase K buffer containing 117 µl RIP Wash Buffer, 15 µl 10% SDS and 18 µl 10 mg/ml proteinase K at 55˚C for 30 min. RNA was isolated and purified from the precipitate using TRIzol® reagent according to the manufacturer's protocol. RT-qPCR was performed to measure the expression levels of NEAT1 and mir-874-3p.

Statistical analysis. All statistical analyses were performed using the SPSS 20.0 software (IBM Corp., SPSS). All results are expressed as the mean ± standard deviation (SD) from at least three independent experiments. Unpaired Student's t-test and one-way ANOVA followed by the Tukey's post hoc test or Pearson's correlation analysis were applied to analyze the significant differences, as appropriate. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of NEAT1 and miR-874-3p in EAFs and PAFs. The expression levels of NEAT1 and miR-874-3p in HF, EAFs and PAFs were detected using RT-qPCR. The results displayed that the expression level of NEAT1 was significantly increased in EAFs and PAFs compared with HF (Fig. 1A). However, the miR-874-3p expression level was significantly downregulated in EAFs and PAFs tissues (Fig. 1B). These results suggested that NEAT1 and mir-874-3p may be involved in follicular atresia progression.

Effects of NEAT1 knockdown on estradiol synthesis. The effect of NEAT1-knockdown on estradiol synthesis using ELISA assay was investigated. The results demonstrated that suppression of NEAT1 significantly increased the production of E2 and P4 in mGCs compared with the si-NC group (Fig. 3A and B). The expression of two genes encoding steroidogenic enzymes, CYP1B1 and CYP19A1, two key genes in estradiol synthesis (25) were also analyzed in mGCs (Fig. 3A and B). The expression of NEAT1 was significantly increased in estradiol synthesis using RT-qPCR. The results revealed that knockdown of NEAT1 significantly decreased the NEAT1 expression of mGCs (Fig. 2A). Furthermore, the MTS assay demonstrated that NEAT1 depletion significantly increased the proliferation of mGCs cells after 48 h compared with the si-NC group (Fig. 2B). Finally, the flow cytometry results showed that the knockdown of NEAT1 significantly decreased the apoptosis ratio in mGCs (Fig. 2C).

Table I. Primers for reverse transcription-quantitative PCR analysis.

| Target gene | Sequence (5’-3’) |
|-------------|-----------------|
| NEAT1-F     | TGAGTAGTGGAAAGCAGGAGAT | |
| NEAT1-R     | GAGGGAAGGAGAGCGAGAGACGA | |
| CYP19A1-F   | GACACATCATGCCTGGAGACC | |
| CYP19A1-R   | GAGATTCCTTGAGACATGTTTT | |
| CYP1B1-F    | CACTTATACCACTTCGTCCG | |
| CYP1B1-R    | AGATGCAGCCGTGCTCACCTT | |
| GAPDH-F     | ATCTGGAGGCTGTGTGATCATACTTCT | |
| GAPDH-R     | GAACCTCACTCTGAGAGATGGT | |
| miR-874-3p-F| CATTTTTTCTACTCTTCTTCTC | |
| miR-874-3p-R| ATCCTTGAGGCTGTGTGATCATACTTCT | |
| U6-F        | CTCGCTTCCGGACGACATATAC | |
| U6-R        | ACGCCCTCAGAATTTGCCGTC | |

F, forward; R, reverse; NEAT1, nuclear-enriched abundant transcript 1; CYP19A1, cytochrome P450 family 19 subfamily A member 1; CYP1B1, cytochrome P450 family 1 subfamily B member 1.

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MiR-874-3p is negatively regulated by NEAT1 in mGCs. Accumulating evidence has suggested that cytoplasmic lncRNAs exert their biological functions by acting as sponges
for miRNAs to negatively regulate miRNAs expression (26). The present study revealed that NEAT1 was mainly located in the cytoplasm of mGCs (Fig. 4A). The online software ENCORI was used to identify the potential miRNA targets of NEAT1. The screening results revealed that miR-874-3p could bind to complementary sequences in NEAT1 (Fig. 4B). Subsequently, luciferase reporter assay was performed to investigate whether miR-874-3p can bind with NEAT1 via direct binding effects. As presented in Fig. 4C, overexpression of miR-874-3p significantly decreased the luciferase activity of WT-NEAT1, but not that of MUT-NEAT1. Furthermore, RIP assay was applied on mGC extracts to verify the direct association between NEAT1 and miR-874-3p using antibodies against Ago2. The results demonstrated that NEAT1 and miR-874-3p were significantly enriched in Ago2 pellets compared with control IgG (Fig. 4D). Additionally, NEAT1 downregulation significantly increased
Figure 3. Knockdown of NEAT1 affects the concentrations of estradiol and progesterone in mGCs. The (A) E2 and (B) P4 concentrations were measured in mGCs transfected with the si-NC or si-NEAT1 using ELISA. The (C) CYP1B1 and (D) CYP19A1 mRNA expression levels were analyzed in mGCs transfected with si-NC or si-NEAT1 using reverse transcription-quantitative PCR. **P<0.01. mGCs, mouse granulosa cells; NEAT1, nuclear-enriched abundant transcript; CYP1B1, cytochrome P450 1B1; CYP19A1, cytochrome P450, family 19, subfamily a, polypeptide 1; E2, estradiol; P4, progesterone; si-, short interfering-; NC, negative control.

Figure 4. miR-874-3p is negatively regulated by NEAT1 in mGCs. (A) Expression of NEAT1 was examined in cytoplasm and nuclear of mGCs by RT-qPCR. (B) WT and MUT binding sites of miR-874-3p on NEAT1 are shown. (C) Luciferase activity was determined in mGCs following co-transfection with miR-874-3p/miR-NC mimics and WT-NEAT1 or MUT-NEAT1 reporter plasmid. (D) Association between NEAT1 and miR-874-3p using RIP assay. (E) Relative expression levels of miR-874-3p were examined by RT-qPCR in mGCs cells transfected with si-NEAT1 or si-NC. (F) Relative expression level of NEAT1 was examined using RT-qPCR in mGCs cells transfected with miR-874-3p mimics or miR-NC. **P<0.01. mGCs, mouse granulosa cells; NEAT1, nuclear-enriched abundant transcript; RT-qPCR, reverse transcription-quantitative PCR; NEAT1, nuclear-enriched abundant transcript 1; MUT, mutant; WT, wild-type; IgG, immunoglobulin G; Ago2, argonaute RISC catalytic component 2, miR, microRNA; NC, negative control; si-, short interfering-.
the expression levels of miR-874-3p in mGCs (Fig. 4E), while overexpression of miR-874-3p significantly decreased the expression levels of NEAT1 in mGCs (Fig. 4F). Overall, these results suggested that NEAT1 acted as a sponge for decreasing miR-874-3p expression.

NEAT1-knockdown affects mGCs proliferation and estradiol synthesis by regulating miR-874-3p. RT-qPCR analysis demonstrated notable miR-874-3p upregulation following transfection with miR-874-3p mimics and downregulation after transfection with miR-874-3p inhibitors (Fig. 5A and B). To verify whether NEAT1 regulated mGCs proliferation and estradiol synthesis by targeting miR-874-3p, a rescue experiment was subsequently conducted. It was revealed that the inhibition of miR-874-3p significantly reversed the NEAT1 knockdown-induced increase of miR-874-3p expression levels in mGCs (Fig. 5C). In addition, miR-874-3p inhibition significantly rescued the effects of NEAT1 depletion on mGCs apoptosis and proliferation, as well as the production of E2 and P4 (Fig. 5D-G). The aforementioned data suggested that NEAT1 regulated mGCs proliferation and estradiol synthesis by sponging miR-874-3p.

Discussion

Several lncRNAs have been identified to be key regulators of the normal development of GCs and are thereby involved in both physiological conditions and pathological processes, such as human oocyte maturation, fertilization, embryo development and ovarian failure (12,13). For example, lncRNA NORHA significantly induces GC apoptosis by influencing the activities of the miR-183-96-182 cluster and the forkhead box protein O1 axis (27). The LINC00477/miR-128 axis contributes to the progression of polycystic ovary syndrome by regulating ovarian granulosa cell proliferation and apoptosis (28). LncRNA steroid receptor RNA activator increases cell growth, inhibited apoptosis and induced secretion of E2 and P4 in mGCs (29). Metastasis-associated lung adenocarcinoma transcript 1 regulates mGC apoptosis and the secretion of E2 and P4 by regulating the miR-205/CREB1 axis (30). The
present study revealed that knockdown of NEAT1 promoted cell proliferation, inhibited apoptosis and induced secretion of E2 and P4 by sponging miR-874-3p, thus suggesting that NEAT1 might be involved in follicular atresia.

GC proliferation and gonadal steroid hormones play key roles in both normal reproductive processes and some reproductive and nonreproductive pathology, including follicular atresia (6,7). GC apoptosis has been confirmed to be a main reason for follicular atresia (2). Thus, understanding of the underlying molecular mechanisms controlling steroid production, cell proliferation and apoptosis within GCs is needed for controlling follicular atresia. In the present study, the results demonstrated that the basal expression levels of NEAT1 in EAFs and PAFs were significantly upregulated compared with HFs. The results also demonstrated that NEAT1 depletion significantly increased cell proliferation, inhibited apoptosis and promoted the production of E2 and P4 in mGCs, thus implying that NEAT1 could participate in follicular atresia progression.

LncRNA NEAT1 has been revealed to function as an oncogene in ovarian carcinogenesis and serves as a potential biomarker for this disease (14,15). Moreover, NEAT1 depletion is able to affect human ovarian GC proliferation and apoptosis through regulation of the miR-381/IGF1 axis (16). However, its functional roles and regulatory mechanisms in GC remain largely unclear. The present study discovered that knockdown of NEAT1 significantly increased cell proliferation and inhibited apoptosis in mGCs, which was consistent with previous results (16). Notably, the present study’s results also revealed that NEAT1 downregulation significantly promoted the production of E2 and P4 in mGCs.

Accumulating evidence has suggested that IncRNAs exerts diverse biological outcomes by sponging miRNAs involved in normal development and pathological responses (25,26,31). Based on the basic principles of interactions between miRNAs and IncRNAs, the underlying mechanism of NEAT1 affecting GC proliferation and apoptosis was subsequently investigated in the present study. The bioinformatical analysis indicated that NEAT1 could bind with multiple miRNAs. MiR-874-3p has been shown to be downregulated and serves as a tumor suppressor in several types of cancer, including ovarian cancer (32). Notably, miR-874-3p has been reported to promote testosterone-induced GC apoptosis by suppressing histone deacetylase 1-mediated p53 deacetylation (33). Furthermore, the luciferase reporter activity and RIP assays in the present study confirmed this interaction. NEAT1 knockdown increased the expression level of miR-874-3p in mGCs, while overexpression of miR-874-3p decreased the expression level of NEAT1. The effects of NEAT1 depletion on mGCs proliferation, apoptosis and the production of E2 and P4 were partially reserved following miR-874-3p inhibition. Taken together, the aforementioned results indicated that NEAT1 regulated mGC proliferation and estradiol synthesis by sponging miR-874-3p.

In summary, the present study demonstrated that NEAT1 functioned as an important regulator of E2 release, cell proliferation and apoptosis in mGCs by sponging miR-874-3p. Although further studies are needed to study other miRNAs that bind with NEAT1 to clarify the regulatory mechanism of NEAT1, the present study demonstrated that NEAT1 can regulate GC cell proliferation and steriodogenesis, suggesting that NEAT1 has therapeutic implications for the control of follicular atresia and fertility.

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

Authors’ contributions
LZ and XLi conceived the study. PZ, JG, SL and GL performed the experiments and wrote the manuscript. WW and CT analyzed the data. XLiu interpreted data. PZ and GL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethical approval and consent to participate
The present study was approved by the Animal Ethics Committee of Jilin Academy of Agricultural Sciences (approval no. JNK20210719-2; Changchun, China) and was in accordance with the Declaration of Helsinki (2000).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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