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MiRNA-let-7b decreases proliferation activities and development of follicular cells via targeting MAP3K1 gene

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ABSTRACT

To date, it has not yet been determined if the apoptosis of follicular granulosa cells (FGCs) is mediated by miR-let-7b via MAP3K1. In the present study, FGCs were transfected with a miR-let-7b mimic at different doses (0, 40, 60, 80, 100 and 120 μM), and were allocated into the control group (CG), and MIM-1, MIM-2, MIM-3, MIM-4 and MIM-5, respectively. Expression levels of miR-let-7b and mitogen-activated protein kinase kinase kinase 1 (MAP3K1) mRNAs and proteins were determined using RT-PCR and Western blots. Luciferase report assay was applied to verify the targeting relationship between miR-let-7b and MAP3K1. The results revealed that the proliferation activity of FGCs in the MIM-4 group was significantly lower than that of the CG and MIM-1 groups ($P<0.05$). The MiR-let-7b mimic obviously reduced expression levels of miR-let-7b of the FGCs. The largest reduction was found in MIM-4. Levels of MAP3K1 mRNAs and proteins in the MIM-3 and MIM-4 groups were lower than that of the CG ($P<0.05$ or $P<0.01$). Co-transfection of let-7b mimic significantly inhibited luciferase activity ($P<0.05$) as compared with the CG. In conclusion, miR-let-7b may obviously depress the cell viability and accelerate apoptosis of ovine FGCs. Higher doses of miR-let-7b mimic (80 μM and 100 μM) could significantly depress expressions of miR-let-7b mRNAs, MAP3K1 mRNAs and protein in ovine FGCs. MiR-let-7b promoted FGCs apoptosis by inhibiting the MAP3K1 gene.

Key words: miRNA-let-7b; follicular granulosa cells; MAP3K1; protein; expression

Introduction

Follicular granulosa cells (FGCs) have a strong ability to proliferate and provide nutrients and regulatory information for the development of oocytes (KOMATSU and MASUBUCHI, 2018, OI et al., 2015). Apoptosis of FGCs is a crucial factor during follicular atresia and folliculogenesis of mammals (TALEBI et al., 2018, YILMAZ et al., 2018). An earlier study showed that follicular atresia occurred when the FGCs were weakened or apoptotic (MUNAKATA et al., 2016, TU et al., 2019). However, the precise functional role of FGC apoptosis in the follicular atresia in sheep remains poorly understood (CAO et al., 2015a, CHENG et al., 2017, YERUSHALMI et al., 2018).

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MicroRNAs (miRNAs) regulated translation of various target mRNAs, involving many cellular and developmental processes (SANG et al., 2015, GEBREMEDHN et al., 2016, TU et al., 2019). The miR-let-7 family is highly conserved across animal species. MiR-let-7a, miR-let-7b, miR-21 and miR-125b were the most abundant miRNAs in ovine follicles, accounting for 40% of all miRNAs (TIAN et al., 2018). It was pointed out that miR-let-7g induced FGC apoptosis by targeting MAP3K1 (mitogen-activated protein kinase kinase kinase 1) in the porcine ovary (CAO et al., 2015a). However, the role miR-let-7 in somatic cells has been scarcely studied (SU et al., 2012, LAN et al., 2019). Meanwhile, it has not yet been determined if FGC apoptosis is mediated by the miR-let-7 family via MAP3K1 in ewes and other females (HAN et al., 2018, CHEN et al., 2019, WANG et al., 2020). The regulatory mechanisms of miR-let-7b in ovine FGC apoptosis during follicle atresia have not been clearly elucidated (LAN et al., 2019).

On the basis of the aforementioned documents, we speculated that miR-let-7b might regulate FGC apoptosis and affect folliculogenesis by targeting MAP3K1. In the present study, we detected expression changes of proliferation activity of ovine FGCs. The levels of miR-let-7b mRNA and proteins of MAP3K1, which was predicted as a targeting gene of miR-let-7b, were determined using qRT-PCR and Western blotting, after the FGC had been treated with varying doses of miR-let-7b mimic, so as to elucidate the regulatory effects of miR-let-7b on FGCs apoptosis, and also to determine whether miR- let-7b modulates FGC apoptosis by via MAP3K1.

Materials and methods

Collection of granulose cells and in vitro culture. The ovaries were aseptically collected from pubescent sheep (6 to 7 months of age) in a local slaughterhouse, placed in DPBS at 37 °C with 100 IU/mL penicillin and 100 μg/mL streptomycin, and immediately brought to the lab in a heated box. Each ovary was punctured with a syringe needle to harvest the 3-5 mm follicles. Follicular cell fluids were collected and centrifuged at 1000 g for 8 min. The supernatants were added to the cell culture medium. The cells were blown evenly. Finally, granulosa cells (1×10^5/mL) were cultured in a 4-well plate using Dulbecco's modified Eagle's medium (EMDM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) added with 10% newborn bovine serum (FBS; GIBCO, Grand Island, NY, USA) and 1% penicillin-streptomycin solution (Gibco; Thermo Fisher Scientific, Inc.), and maintained in a humidified atmosphere containing 5% CO_2 at 37.0 °C.

All experiments in this study were conducted strictly following the guidelines of the Animal Care and Use Committee of the Animal Ethics Committee of Northwest Minzu University, China (approval number: XBMU-2015-016).

FGCs transfection with miR-let-7b mimic. Follicular granulosa cells (FGCs) were seeded into 4-well plates for 24 h. FGCs were transfected with miR-let-7b mimic (RiboBio Biotech Co., Ltd; Guangzhou, China) at different doses of 0 μM, 40 μM, 60 μM, 80 μM, 100 μM and 120 μM, and these were allocated into the control group (CG), and MIM-1, MIM-2, MIM-3, MIM-4 and MIM-5 groups respectively, following the manufacturer's protocol for Lipofectamine 2000 Reagent (Invitrogen, Cat. No.11668-019, Carlsbad, CA, USA), for 72 h. At 24, 48 and 72 hours after transfection, FGCs were collected for an assay using the Cell Counting Kit-8 (CCK-8). FGCs transfected with Lipofectamine 2000 only were used as the control group (CG).

Proliferation activity assays of FGCs. Cell proliferation activity was assessed using the Cell Counting Kit-8 assay (CCK-8; Dojindo, Kumamoto Japan) according to the manufacturer's instruction. FGCs that were transfected with miR-let-7b mimic and NC were seeded into 96-well plates to determine the influence of miRNAs on proliferation activity. All cells were cultured for 72 h. CCK-8 solution was then added to each well according to the protocol of the manufacturer. After 3 h of incubation, the absorbance was read at 450 nm using an Elx800 Reader (Bio-Tek Instruments Inc., USA). This assay was conducted in triplicate.

According to the calculation formula for the CCK-8 kit, the proliferation activities of granulosa cells were calculated for each group.

Apoptosis assays. FGCs transfected with let-7b mimic and NC were harvested after 24 h, 48 h and 72 h of transfection, respectively. The apoptosis effect was measured using the Annexin V-FITC Apoptosis Detection Kit (KeyGEN, Nanjing, China). Briefly, FGCs were cultured in 6-well trays and transfected with plasmids for 48 h. Then, 500 μL of 1X Annexin V buffer was added to gently resuspend the cells, and 5 μL of Annexin V-FITC and 5 μL of propidium iodide staining solution were added and mixed. Flow cytometry was performed after incubation for 15 min at room temperature. Apoptosis cells were quantified by flow cytometry according to the manufacturer's instructions. The value of FGC apoptosis was the ratio between the miR-let-7b treatment group the and control group (CG).

Quantitative real-time PCR (qRT-PCR) of miR-let-7b and MAP3K1 genes

Primer design. For qRT-PCR, the specific primers were chosen in well-conserved regions of the miR-let-7b mimic (NR-107877.1; MI-0014113), miR-U6 (Gene ID: 108353825; Gene Accession: NC-015438.3), MAP3K1 (Gene Accession: NM-001205906.1; NP-001192835.1) and sheep actin-beta (Gene Accession: NM-001009784.3; DQ386889.1) (Table 1).

RNA extraction and cDNA synthesis. FGCs were cultured for 72 h, and washed twice with DPBS. Total RNA was extracted by lysing granulosa cells in TRIzol reagent (Invitrogen; Carlsbad, CA, USA), according to the manufacturer's instructions. The RNA concentration was determined by absorbance at 260 nm using UV-2600i, UV-2700i ultraviolet spectrophotometer (Daojin Co. Ltd, Shanghai, China). 1 μg of total RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara; Dalian, China) according to the manufacturer's instructions.

qRT-PCR. qRT-PCR was performed with a SYBR Premix Ex Taq™ II kit (Takara; Dalian, China) using a CFX96 real-time PCR system (Bio-Rad; Hercules, CA, USA). The reaction was incubated for pre-denaturation at 95 °C for 10 min, 40 cycles of denaturationat 95 °C for 30 s and annealing/elongation at 60 °C for 55 s. Fluorescence signals were collected during the annealing step. U6 and β-actin were employed as endogenous controls for MiR-let-7b and MAP3K1, respectively. The relative gene expression levels were calculated with reference to the expression of the U6 and β-actin using the $2^{-\Delta\Delta Ct}$ method. All of the reactions were performed in triplicate.

Western blots of MAP3K1 proteins in granulosa cells. To determine the expression levels of MAP3K1 proteins, FGCs were harvested from culture plates, lysed in RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.1% SDS, 2mM EDTA, 1mM PMSF, 1% NP40, 5 μg/mL aprotinin,

Table 1. Primers used for qRT-PCR of miR-let-7b and MAP3K1 gene

Primer	5' to 3'	Length (bp)
miR-let-7b mimic-F	UGAGGUAGUAGGUUGUGUGGU	21
miR-let-7b mimic-R	CACACAACCUACUACCUCAUU	21
miR-U6-F	CTCGCTTCGGCAGCACA	17
miR-U6-R	AACGCTTCACGAATTTGCGT	20
MAP3K1-F	TTATCGGGCCTCAGAACTGC	20
MAP3K1-R	ATGGTGTTACGAGACGGAGC	20
Ovine β-actin-F	CCAAAGCCAACCGTGAGAA	19
Ovine β-actin-R	AGAGGCGTACAGGGACAGCA	20

and 1 μg/mL leupeptin) on ice, and then centrifuged at 12000×g for 10 min.

Total protein was extracted from the FGCs using a total protein extraction Kit (Applygen Technologies, Beijing, China) according to the manufacturer's instructions. Protein concentrations were determined with a BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Haumen, China). Proteins were loaded onto 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to polyvinylidene fluoride (PVDF) membranes and blocked in 5% non-fat milk in 10 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, 0.1% (w/v) Tween 20 for 2 h. The polyclonal antibody to MAP3K1 (Boster Biological technology, Pleasanton, USA; 1: 2000) was diluted and incubated at 4 °C overnight, followed by 1 h of incubation with the appropriate secondary antibody (1:500, Goat Anti-Mouse IgG). The photo was scanned with a gel image analyzer. The absorbance (A value) of each strip was analyzed using Quantity One software. The ratio of the absorbance of the proteins strip to the absorbance of the β-actin strip indicated the relative amount of proteins in the FGCs. All experiments were performed in triplicate.

Target gene predictions of miR-let-7b. To investigate the possible mechanism of miR-let-7b further by regulating MAP3K1 expression, miRanda (http://www.microrna.org/) and TargetScan (USA) were used to predict whether MAP3K1- miRNAs is related to miR-let-7b.

Co-transfection of 293T cells

Construction of psiCHECK2-MAP3K1-3'UTR expression vectors. The total RNA of 293T cells was extracted using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The cDNA was prepared via reverse transcription. The MAP3K1-3′UTR was amplified from the cDNA using PrimeSTAR® Max DNA polymerase (Takara Bio, Inc., Otsu, Japan) and cloned into psiCHECK2 vector (Invitrogen; Thermo Fisher Scientific, Inc.) to construct psiCHECK2-MAP3K1-3′UTR-wild type (WT). The MAP3K1-3′UTR primer sequences (NM_001205906.1; GI: 329663887) were as follows: Forward: 5′- tgtgttgtgtttcctctttttttg -3′; Reverse: 5′-ctttgttcaaaaacttttatttactataaag-3′. The thermocycling conditions were as follows: 94 °C for 3 min, 94 °C for 30 s, 58 °C for 30 s, 72 °C for 102 s and 72 °C for 5 min. Wide type and mutant type psiCHECK2-MAP3K1-3′UTR vectors were transfected with either miR-let-7b mimic or NC.

Co-transfection of 293T cells with miR-let-7b mimic WT or MU. MiR-let-7b mimic (or its negative control, NC), and the wild-type (WT) and mutant-type (MT) of MAP3K13′-UTR were synthesized and purchased, respectively, from RiboBio Co., Ltd (Guangzhou, China). They were inserted into the multiple cloning sites of the luciferase expressing psiCHECK2 vectors (Ambion; Thermo Fisher Scientific, Inc.). In this experiment, two vectors were successfully constructed, including the MAP3K1-WT+miR-let-7b mimic vector (containing the wild-type miR-let7b binding site sequence) and the MAP3K1-MU+miR-let-7b mimic vector (containing part of the miR-let7b binding site sequence). Both MAP3K1-WT and MAP3K1-MU miR-let-7b vectors were co-contransferred into 293T cells, respectively, with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc) for 48 h at 37 °C according to the manufacturer's protocol. After 48 h of transfection, 293T cells were collected and measured using dual-luciferase assay. Blank 293T cells were used as the control group.

Before co-transfection, 293T cells were digested by trypsin, accessed to 4-well plates, 5% CO_2, and then cultured in opti-MEM media (Thermo Fisher Scientific, Shanghai, China) with 10% FBS, 1% penicillin-streptomycin and 1% glutamine in a humidified atmosphere containing 5% CO_2 at 37.0 °C for 24 h.

Dual luciferase assay. The dual luciferase assay was performed using a Dual-Luciferase® Reporter Assay System (Promega Corporation, Madison, WI, USA). Briefly, 293T cells were cultured in 24-well plates and co-transfected with MAP3K1-WT+miR-let-7b mimic, MAP3K1-MU+ miR-let-7b mimic (or its NC) and Renilla luciferase vectors, respectively, using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h, cells were collected and the dual-luciferase activity was determined using the Dual Luciferase Reporter System (Promega; Madison, WI, USA). Renilla luciferase was used as the internal control. The

results were presented as ratios between firefly and Renilla activity. The experiments were performed independently in triplicate.

2.10 Statistical analyses. Statistical analysis was performed using IBM SPSS Statistics version 21.0 (SPSS Inc., USA) and GraphPad Prism Version 7.0 (GraphPad Software, Inc.; La Jolla, CA, USA). Data were presented as the means ± SE. The Student's t-test was used to analyze differences between the different treatment groups. Inter-group differences were analyzed by one-way analysis of variance, followed by a post hoc Tukey test for multiple comparisons. A P value less than 0.05 was considered to indicate a statistically significant difference.

Results

miR-let-7b decreased FGC proliferation activities. The proliferation activities of FGCs were gradually decreased in line with rising miR-let-7b mimic dosage (from 40 μM to 120 μM) (Fig. 1). The proliferation activities of FGCs in MIM-4 and MIM-5 were significantly less than in the CG ($P<0.01$). The maximum decrement was detected in the MIM-4 (100 μM) group, which was significantly lower than those of the CG and MIM-1 groups ($P<0.05$ or $P<0.01$). The outcomes indicated that miR-let-7b mimic (100 μM and 120 μM) may signficantly reduce the proliferation activities of FGCs.

MiR-let-7benhanced FGCs apoptosis. The results showed the apoptosis ratio of the miR-let-7b mimic groups were increased in line with the increase in miR-let-7b mimic doses as compared to the CG (Fig. 2). The maximum increment was found in the MIM-4 group. The apoptosis value of the MIM-4 group was greater than those of the CG and MIM-1 groups. The results indicate that 100 μM of miR-let-7b mimic could strongly promote apoptosis of ovine FGCs.

Expression levels of miR-let-7b mRNAs in FGCs. As compared to the control group (CG), the expression level of miR-let-7b in the FGCs was obviously reduced (Fig. 3). The maximum reduction was detected in the MIM-4 group ($P<0.01$). Additionally, miR-let-7b levels of the MIM-3 and MIM-4 groups were less than that of MIM-1 ($P<0.05$ or $P<0.01$). These results demonstrated that a high dose (100 and 120 μM) of miR-let-7b mimic treatment could depress the expression levels of miR-let-7b mRNAs in the FGCs.

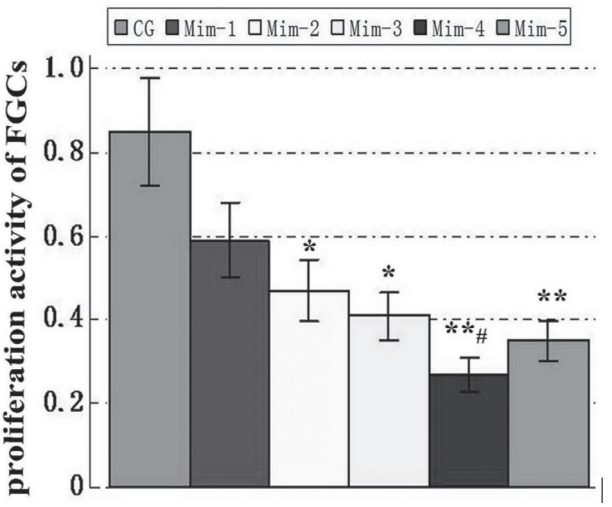

Fig. 1. Proliferation activity of FGCs

Control Group (CG) - 0 μM miR-let-7b mimic, MIM-1 - 40 μM miR-let-7b mimic, MIM-2 - 60 μM miR-let-7b mimic, MIM-3 - 80 μM miR-let-7b mimic, MIM-4 - 100 μM miR-let-7b mimic and MIM-5 - 120 μM miR-let-7b mimic. $^{*}P<0.05$, as compared to the CG (0 μM miR-let-7b mimic), $^{**}P<0.01$, as compared to the CG (0 μM miR-let-7b mimic); $^{\#}P<0.05$ as compared to the mimic 1 group (MIM-1, 40 μM miR-let-7b mimic).

MiR-let-7b directly targeted the 3′UTR of MAP3K1 mRNA.

Bioinformatic prediction of the target genes of miRNA and their binding sites revealed that one conserved domain in the 3-UTR of MAP3K1 could bind with miR-let-7b. MAP3K1 was identified as a target gene of miR-let-7b.

Expression levels of MAP3K1 mRNAs and proteins in FGCs. Expression levels of MAP3K1 mRNAs in the FGCs were significantly changed after FGCs were treated with different doses of miR-let-7b mimic (Fig. 4). Levels of MAP3K1 proteins were altered very similar to those of MAP3K1 mRNAs in FGCs.

The miR-let-7b mimic dose-dependently (from 40 to 100 μM) declined expression levels of MAP3K1 mRNAs and proteins. Levels of MAP3K1

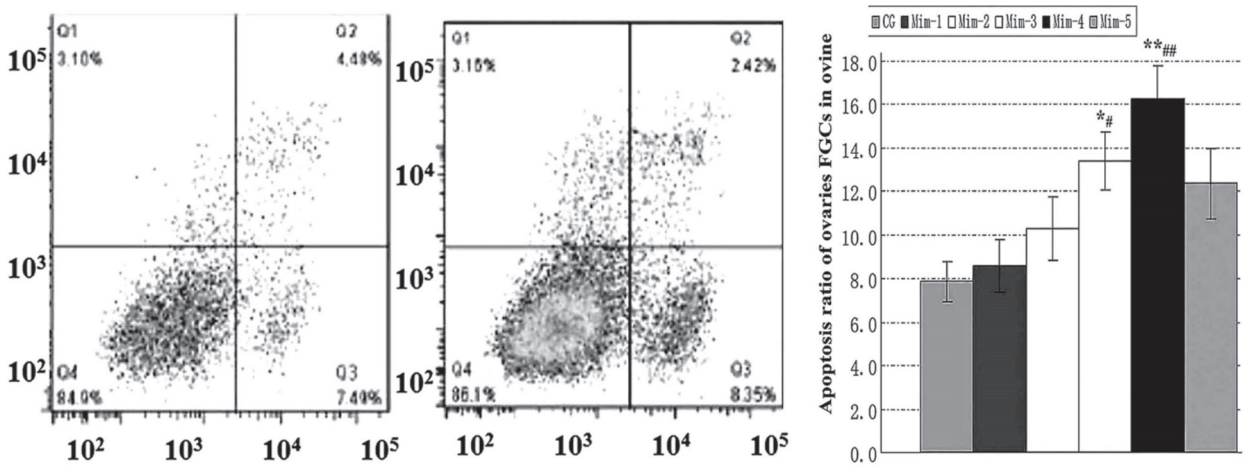

Fig. 2. Apoptosis detection of ovarian FGCs using Annexin-V/PI staining and flow cytometry analysis

Control Group (CG) - 0 μM miR-let-7b mimic, MIM-1 - 40 μM miR-let-7b mimic, MIM-2 - 60 μM miR-let-7b mimic, MIM-3 - 80 μM miR-let-7b mimic, MIM-4 - 100 μM miR-let-7b mimic and MIM-5 - 120 μM miR-let-7b mimic. * P<0.05, as compared to CG (0 μM miR-let-7b mimic), **P<0.01, as compared to the CG (0 μM miR-let-7b mimic). #P<0.05 as compared to MIM-1 (40 μM miR-let-7b mimic) group, ##P<0.01 as compared to the MIM-1 (40 μM miR-let-7b mimic) group.

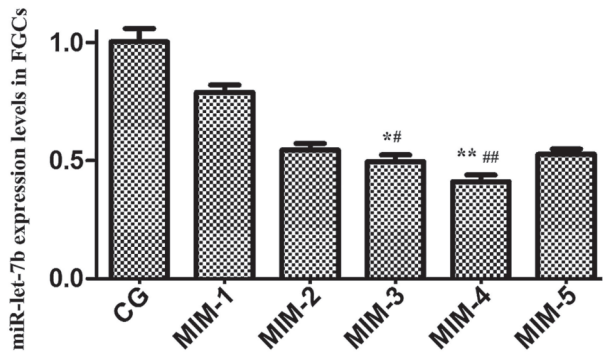

Fig. 3. Expression levels of miR-let-7b mRNAs after the FGCs were treated with miR-let-7b mimic

The miR-let-7b expression levels of FGCs decreased in comparison with the CG. Control Group (CG) - 0 μM miR-let-7b mimic, MIM-1 - 40 μM miR-let-7b mimic, MIM-2 - 60 μM miR-let-7b mimic, MIM-3 - 80 μM miR-let-7b mimic, MIM-4 - 100 μM miR-let-7b mimic and MIM-5 - 120 μM miR-let-7b mimic.Note: The U6 gene was used as a normalization control. * P<0.05, as compared to the CG (0 μM miR-let-7b mimic), **P<0.01, as compared to the CG (0 μM miR-let-7b mimic). #P<0.05 as compared to the MIM-1 (40 μM miR-let-7b mimic) group, ##P<0.01 as compared to the MIM-1 (40 μM miR-let-7b mimic) group.

mRNAs and proteins in the MIM-3 and MIM-4 groups were less than that of the CG (P<0.05 or P<0.01). Additionally, levels of MAP3K1 mRNAs and proteins of MIM-4 group were lower than that of MIM-1 (P<0.05).

The findings demonstrated that higher doses of miR-let-7b mimic (80 μM and 100 μM) could significantly promote or depress expressions of MAP3K1 mRNAs and protein in ovine FGCs.

Construction of psiCHECK2-MAP3K1-3UTR vectors. To explain the assumption further, luciferase reporter assays were conducted in 293T cells. Firstly, psiCHECK2-MAP3K1-3UTR vectors were successfully constructed. They were then transfected into *E*. coli. Additionally, 10 μL of ligated-products were transfected into DH5α competent cells. The results of PCR showed that a specific band was found in 418 bp (data omitted), which was similar to the prediction.

Levels of MAP3K1 proteins in 293T cells

Effects of miR-let-7b transfections on 293T cells. The transfections of 293T cells using wild type and mutant type psiCHECK2-MAP3K1-3UTR vectors and miR-let-7b had distinct efficacy (Fig. 5). The gray values of the wild type of psiCHECK2-MAP3K1-3UTR vector were darker than those of the mutant type.

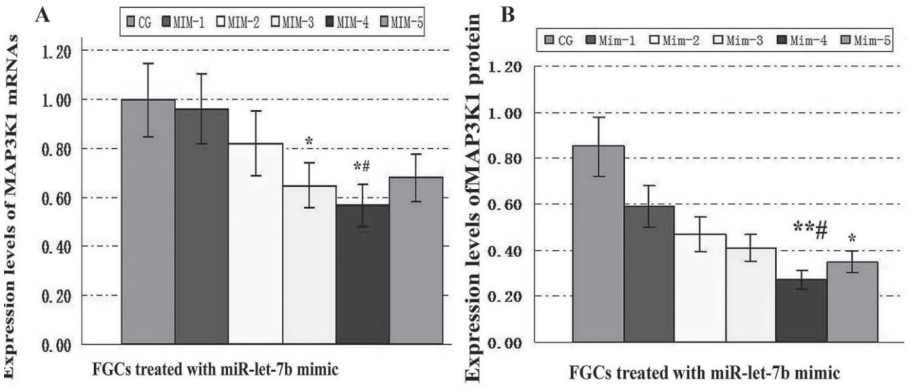

Fig. 4. Expression levels of MAP3K1 mRNAs and proteins in FGCs

Control Group (CG) - 0 μM miR-let-7b mimic, MIM-1 - 40 μM miR-let-7b mimic, MIM-2 - 60 μM miR-let-7b mimic, MIM-3 - 80 μM miR-let-7b mimic, MIM-4 - 100 μM miR-let-7b mimic and MIM-5 - 120 μM miR-let-7b mimic. A. MAP3K1 (mitogen-activated protein kinase kinase kinase 1) mRNAs levels in FGCs transfected with miR-let-7b mimic. B. MAP3K1 proteins levels in FGCs transfected with miR-let-7b mimic. $^{*}P<0.05$ as compared to the CG (0 μM miR-let-7b mimic), $^{**}P<0.01$ as compared to the CG (0 μM miR-let-7b mimic). $^{\#}P<0.05$ as compared to the MIM-1 (40 μM miR-let-7b mimic) group.

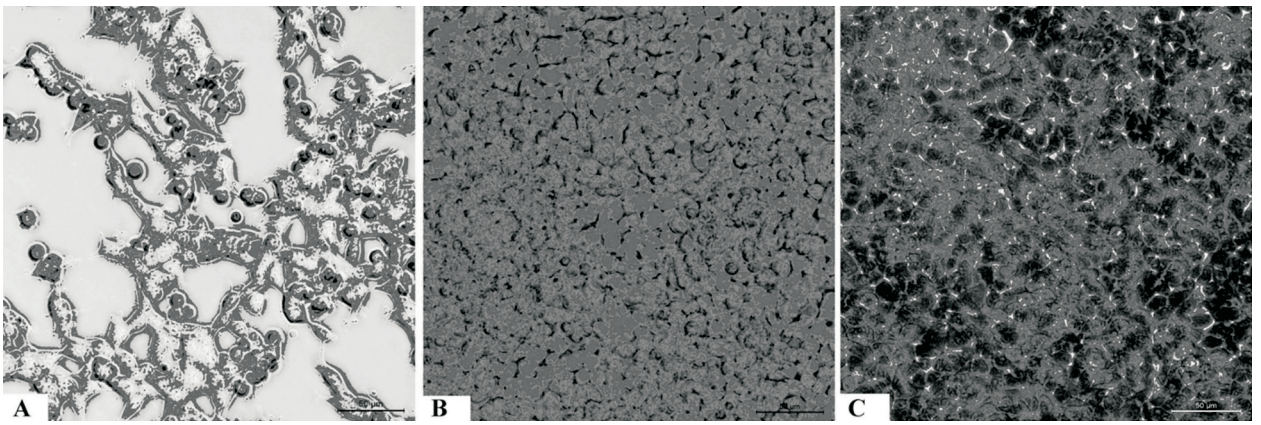

Fig. 5. 293T cells transfected with psiCHECK2-MAP3K1-3UTR vectors（×200）

The gray values of the wild type of psiCHECK2-MAP3K1-3UTR vector were darker than those of the mutant type. A: Wild type; B: Mutant type.

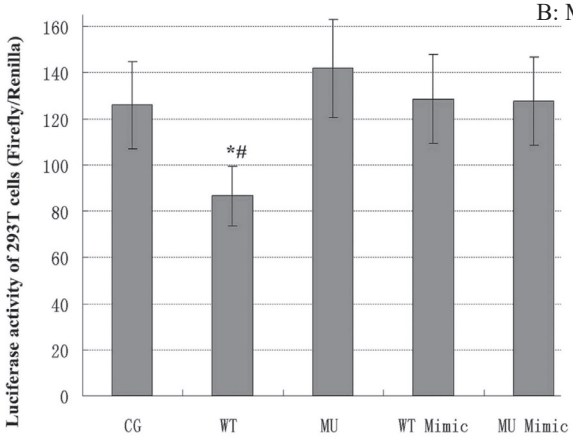

Fig. 6. The luciferase activity of 293T cells

MAP3K1-WT+ miR-let-7b mimic reduced the luciferase activity of 293T cells. However, there was no significant difference in the luciferase activity between the CG and MU mimic NC groups. MAP3K1 was a direct target of miR-let-7b. NC: negative control; WT: wild type; MU: mutant type. $^{*}P<0.05$ in comparison with the CG; $^{**}P<0.01$ in comparison with the CG.

MAP3K1 was a direct target of miR-let-7b

To validate the direct binding site, luciferase reporter assays were used to evaluate luciferase activity. 293T cells were co-contransferred with MAP3K1-WT+miR-let-7b mimic and MAP3K1-MU+ miR-let-7b mimic (or its NC), respectively. Co-transfection of the MAP3K1-WT+ miR-let-7b mimic significantly inhibited luciferase activity when compared with the CG and MAP3K1-MU+miR-let-7b mimic ($P<0.05$) (Fig. 6).

As shown in Fig. 6, there was no significantly difference in the luciferase activity between the CG and MU mimic NC groups. Therefore, the effect was eliminated when the mutation sequences were introduced into their binding sites. These findings demonstrate that MAP3K1 was a direct target of miR-let-7b.

Discussion

Follicular atresia caused by FGC apoptosis mainly affects all stages of follicular growth and development (LIU et al., 2014, FU et al., 2018). Earlier reports indicated that the miR-let-7 family was involved in cell differentiation of tumors by targeting genes (THAMMAIAH and JAYARAM, 2016). However, studies on the role of let-7 in somatic cell processes are limited (TU et al., 2019, ZHANG et al., 2019).

Previous studies showed that the survival rate of porcine oocytes was significantly decreased when porcine granulose cells (GCs) were treated *in vitro* with miRNA mimics (CAO et al., 2015a, KIM et al., 2013). Our results revealed that the survival rate (or proliferation activity) of FGCs gradually decreased along with rising miR-let-7b mimic doses. Proliferation activity in the MIM-4 group was significantly lower than that of the CG and MIM-1 groups. Over-expression of miR-let-7b in FGCs may signficantly depress the proliferation activity. These outcomes were in agreement with previous reports (ZHOU et al., 2015, CAO et al., 2015b).

Concurrently, the present study explored the effects of miR-let-7b on expression levels of miR-let-7b mRNAs in FGCs. The MiR-let-7b mimic clearly reduced expression levels of MiR-let-7b in FGCs. The maximum reduction was detected in the MIM-4 group. A high dose of miR-let-7b mimic (100 μM) may greatly alter the expression levels of miR-let-7b mRNAs in ovine FGCs. These findings resembled initial studies which showed that miRNA mimic attenuated expression levels of miR-let-7b, let-7c and miR-27a in porcine granulose cells (pGCs) (LIU et al., 2014, SUN et al., 2018).

MAP3K1 plays a protective role in cell survival and proliferation, and the cell cycle. Deletion or silencing of MAP3K1 causes cell cycle arrest or cell death. MAP3K1 protein was tested to improve cell survival (PHAM et al., 2013).

The findings of qRT-PCR and Western blotting indicated that miR-let-7b mimic dose-dependently decreased expression levels of MAP3K1 proteins in FGCs. The level of MAP3K1 proteins in the MIM-4 group was lower than that of MIM-1. These outcomes demonstrated that miR-let-7b could accelerate ovine FGC apoptosis by inhibiting the MAP3K1 gene. MAP3K1 was a direct target of miR-let-7b. The outcomes of the present study are similar to previous reports that overexpression of let-7g inhibits the expression of MAP3K1 of porcine ovary follicle (CAO et al., 2015b) and miR-203 affects esophageal cancer cell proliferation, apoptosis and invasion, by targeting MAP3K1 (ZONG et al., 2020).

293T cells were co-transfected with MAP3K1-WT+miR-let-7b mimic and MAP3K1-MU+ miR-let-7b mimic (or NC), respectively. The luciferase reporter assay confirmed the site on MAP3K1 mRNA targeted by miR-let-7b. The gray values of MAP3K1-WT+miR-let-7b mimic were lower than those of the mutant type and the CG. Co-transfection of WT miR-let-7b mimic significantly inhibited luciferase activity. However, these effects were eradicated when the mutation sequences were introduced into their binding sites. These findings confirmed that MAP3K1 was a direct target of miR-let-7b.

Conclusions

miR-let-7b may significantly depress the cell viability of FGCs. A high dose of miR-let-7b mimic (100 μM) could depress the expression levels of miR-let-7b mRNAs in FGCs. miR-let-7b dose-dependently decreased expression levels of MAP3K1 proteins. MiR-let-7b could accelerate ovine FGC apoptosis by depressing the MAP3K1 gene. Furthermore, we demonstrated that MAP3K1 was a direct target of miR-let-7b, using the dual-luciferase reporter assay in 293T cells. The miR-let-7b is probably a potential target for diagnosing and treating ovarian cancers. These findings may provide a basis for elucidating the regulatory effects of miR-let-7b in the apoptosis of FGCs, and also offer a novel insight for further exploration of the molecular regulation of the miR-let-7 family and MAP3K1 on FGC functions in folliculogenesis in female mammals.

Conflict of i nterest

The authors declare no conflict of interest.

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Authors' contributions

Miss Yang Juan carried out the Western assay. Professor Gong Zhuandi designed the experiments and carried out the cell culture. Mr. Bai Xiaoqiang was responsible for the initial design. Miss Bai Shengju measured the detected receptor gene expression. Mr. Yu Hui performed the luciferase assay. Professor and Dr. Wei Suocheng designed the experiments and wrote the manuscript. All authors interpreted the data, critically revised the manuscript for important intellectual content, and approved the final version.

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SAŽETAK

Do danas nije definirano je li apoptoza folikularnih granuloza-stanica (FGCs) posredovana prekursorom miR-let-7b putem gena MAP3K1. U ovom je istraživanju FGC transfeciran miR-let-7b imitatorom u različitim dozama (0, 40, 60, 80, 100 i 120 μM) i potom razvrstan u kontrolnu skupinu (CG), te u skupine označene kao MIM-1, MIM-2, MIM-3, MIM-4 i MIM-5. Razine ekspresije mRNA i proteina miR-let-7b i mitogenom-aktivirane proteinske kinase kinase kinase1 (MAP3K1) određene su uporabom RT-PCR-a i Western blot-a. Test luciferaze je primijenjen kako bi se potvrdio ciljni odnos između miR-let-7b i MAP3K1. Rezultati su pokazali da je proliferacijska aktivnost FGC-a u skupini MIM-4 bila znakovito manja nego u kontrolnoj skupini i MIM-1 skupini ($P<0,05$) što ide u prilog pretpostavci da miR-let-7b imitator može u FGCs smanjiti razinu ekpresije miR-let-7b. Najveće smanjenje utvrđeno je u skupini MIM-4. Razine MAP3K1 mRNAs i proteina u skupinama MIM-3 i MIM-4 bile su niže nego u kontrolnoj skupini ($P<0,05$; $P<0,01$). Transfekcija let-7b imitatorom znakovito je inhibirala aktivnost luciferaze ($P<0,05$) u usporedbi s kontrolnom skupinom. Zaključno, miR-let-7b može smanjiti opstojnost stanice i ubrzati apoptozu ovčjih FGC-a. Veće doze miR-let-7b mimic (80 μM i 100 μM) mogu znakovito smanjiti ekspresiju miR-let-7b mRNAs, MAP3K1 mRNAs i proteina u ovčjih FGC-a. MiR-let-7b putem inhibirajućeg gena MAP3K1 doprinosi apoptozi folikularnih granuloza-stanica (FGCs).

Ključne riječi: miRNA-let-7b; folikularne granuloza-stanice; MAP3K1; protein; ekspresija