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Study on the expression patterns and biological functions of *oar-miR-214_3p* in the ovary and uterus of Liangshan Black Sheep

*Weihua Chang[1,2], Fengjiao Qiu[1], Shanchuan Liu[1], Guochao Ni[1], Ying Zhao[1], Yi Zhang[3], Guangwen Yan[1], Juanhong Wang[1]**

[1]*College of Animal Science, Xichang University, Xichang City, Sichuan, P.R. China*
[2]*Key Laboratory of Animal Epidemic Disease Detection and Prevention in Panxi District of Sichuan, Xichang City, Sichuan, P.R. China*
[3]*Key Laboratory of Local Characteristic Goat of Sichuan Province, Xichang City, Sichuan, P.R. China*

**Corresponding author: wangjuanhonglove@163.com*

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Abstract: This study elucidates the expression dynamics and biological functions of *oar-miR-214_3p* in ovarian and uterine tissues of Liangshan Black Sheep across distinct stages of the oestrous cycle, providing novel insights into its regulatory mechanisms governing ovine reproductive physiology. The study employed RT-qPCR, northern blotting, FISH, dual-luciferase reporter assays, and ELISA to analyse the expression and biological functions of *oar-miR-214_3p* in the ovary and uterus of Liangshan Black Sheep across different stages of the oestrous cycle (proestrus, oestrus, metoestrus, and dioestrus phases). RT-qPCR and northern blotting revealed that *oar-miR-214_3p* is expressed in the ovarian and uterine tissues, with significant variations across different stages. Compared with the dioestrus and metoestrus phases, *oar-miR-214_3p* expression was significantly increased during the proestrus and oestrus phases ($P < 0.05$), with the highest levels observed during oestrus ($P < 0.05$). FISH analysis indicated that *oar-miR-214_3p* is primarily localised in the cytoplasm. We constructed wild-type and mutant vectors for mammalian target of rapamycin(mTOR) and Semaphorin 4D (Sema4D). After intervention for 48 h in granulosa cell cultures, RT-qPCR analysis of *mTOR* and *Sema4D* expression revealed that the mimic and inhibitor groups suppressed and promoted the expression of these target genes, respectively. The control and NC groups showed stable expression levels with no significant differences. Dual-luciferase reporter assay confirmed that *mTOR* and *Sema4D* are *oar-miR-214_3*p target genes. ELISA revealed that the mimic and inhibitor treatment groups promoted and suppressed oestrogen secretion, respectively. These findings confirm that *oar-miR-214_3p*, primarily localised in the cytoplasm, regulates ovarian follicle development, ovulation, oestrogen secretion, and the oestrous cycle via its target genes, *mTOR* and *Sema4D*. The study provides valuable insights into improving sheep reproductive performance through molecular breeding techniques. These findings have significant clinical and practical applications for enhancing reproductive efficiency.

Keywords: cytoplasmic localisation; dual-luciferase assay; folliculogenesis; reproductive behaviour; steroidogenesis

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MiR-214 is a vertebrate-specific miRNA family with a mature sequence of 22 nucleotides generated by the enzyme Dicer. This family plays a significant regulatory role in various biological processes, including cell regulation, embryonic development, reproductive physiology, autophagy, drug resistance, cancer, and coronary artery disease (Karimi et al. 2023; Chen et al. 2024), and exhibits a broad range of functions. The regulatory mechanisms of miR-214 in the reproductive function of Liangshan Black Sheep, a newly discovered local breed from Sichuan Province, remain poorly understood. Exploring the regulatory mechanisms of miR-214 in the areas of ovarian follicle development, ovulation, and hormone secretion in Liangshan Black Sheep is crucial for improving the reproductive efficiency and promoting multiple-lamb sheep development in this breed. Studies by Chang et al. (2016), Na et al. (2022), and Tian et al. (2018) have confirmed that miR-214 is expressed at significantly higher levels in the ovaries than in other organs (testes, uterus, fallopian tubes, pituitary, etc.). Yuan et al. (2015) studied the pituitary miRNAs of 21-day-old and 12-month-old mice and confirmed that miR-214 shows significant differences in expression across different ages of mice. Wu et al. (2017) demonstrated that miR-214 plays a crucial role in the angiogenesis and the differentiation of embryonic stem cells, thus revealing its involvement in the differentiation of embryonic stem cells into vascular smooth muscle cells, with the Quaking Gene identified as a new miR-214 target gene in this process. Tian et al. (2018) investigated ovary tissue from adult pigs with high and low litter sizes and found that miR-214 expression was downregulated in high-fertility pigs, with the lowest expression levels observed on days 14 and 7 of the oestrous cycle. These findings suggest that miR-214 plays a regulatory role in follicular development, ovulation, luteinisation, and oestrus. A number of studies have shown that the ovary is a crucial target organ for miR-214 (Tian et al. 2018; Liu et al. 2023; Wang et al. 2024). The two primary ovary functions, ovulation and hormone secretion, underscore the significance of miR-214 in regulating ovarian physiological functions. However, further studies are needed to fully understand the mechanisms by which *oar-miR-214_3p* regulates these functions. Liangshan Black Sheep is a newly discovered local breed identified in 2021 from Liangshan Yi Autonomous Prefecture in Sichuan Province. This breed is characterised by seasonal oestrus, single births, and excellent traits such as resistance to roughage and strong adaptability to adverse conditions. A previous study revealed that the *oar-miR-214_3p* gene is a vital ovarian miRNA closely associated with oestrus in sheep (Chang et al. 2016). Given that there are limited studies on miRNAs and their role in regulating reproductive physiology in Liangshan Black Sheep, this study aims to explore the temporal and spatial expression of the *oar-miR-214_3p* gene in the reproductive organs and tissues of Liangshan Black Sheep during the proestrus, oestrus, metoestrus, and dioestrus phases. This research contributes to improving sheep reproductive performance through molecular breeding techniques and enhancing reproductive efficiency and thus offers significant clinical and practical value.

MATERIAL AND METHODS

Ethical statement

All animal experiments in this study were approved (No. xcc2023006) by the Ethics Committee and Laboratory Animal Welfare Committee of Xichang University and performed in accordance with the "Guidelines for Use of Experimental Animals" of the Ministry of Agriculture (Beijing, P.R. China).

Experimental animals and sample collection

Twelve healthy female Liangshan Black Sheep, weighing 65 ± 2.5 kg, exhibiting normal oestrous cycles, and experiencing with at least two lambings were selected as experimental sheep. After 30 days of isolation and observation, they were allowed free access to food. The sheep were randomly divided into four groups. Each group underwent synchronised oestrus using the CIDR + PMSG + PG method. The oestrous synchronisation program was as follows: Day 0: insert removal; Day 7: insertion change; Day 14 at 8 a.m.: final insert removal. Subsequently, PMSG 400 IU and PG 0.1 g were injected on the same day. Oestrus was tested on Days 15 and 16 (morning and evening).

Samples were collected from ovarian, uterine, muscle, and other tissues during the proestrus, oestrus, metoestrus, and dioestrus phases (10 days

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after oestrus) and quickly frozen in liquid nitrogen. Furthermore, fluorescence *in situ* hybridisation (FISH) samples were fixed in formaldehyde.

RNA extraction

Approximately 100 mg of tissue was ground in liquid nitrogen using 1 ml RNAiso Plus, transferred to an RNase-free EP tube, and incubated at room temperature for 5 minutes. Centrifugation was performed at 12 000 × *g* for 10 min at 4 °C. The supernatant was carefully transferred to a new 1.5 ml tube.

Subsequently, 0.2 ml of chloroform was added, and then the tube was capped, shaken vigorously for 15 s, and incubated at room temperature for 5 minutes. Next, centrifugation was performed at 12 000 × *g* for 15 min at 4 °C. The aqueous phase was transferred to a new tube, and 0.5 ml of isopropanol was added. The solution was incubated at room temperature for 10 min and centrifuged at 12 000 × *g* for 10 min at 4 °C, and then the supernatant was discarded. In addition, the pellets were washed with 1 ml of 75% ethanol and centrifuged at 7 500 × *g* for 5 minutes. The supernatant was discarded, and the pellets were air-dried at room temperature for 10 minutes. RNase-free water was added according to the amount of pellet, and the RNA concentration was measured using a micro-spectrophotometer. Qualified samples were stored at –80 °C for subsequent experiments.

Reverse transcription and quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Furthermore, 4 µl of 5 × RT SuperMix was added, and the total reaction volume was adjusted to 16 µl. The mixture was gently mixed, and the reaction was performed in a PCR system as follows: 37 °C for 15 min and 85 °C for 5 s to obtain the reverse-transcribed cDNA. The PCR reaction mixture was prepared in an RNase-free microtube containing 10 µl of 2 × S6 Universal SYBR qPCR Mix, 2 µl of cDNA, 0.4 µl of forward and reverse primers (10 µmol/l), 7.2 µl of ddH_2O. The PCR program is as follows (total volume of 20 µl): Step 1: 95 °C for 30 s (1 cycle); Step 2: 95 °C for 10 s and 60 °C for 30 s (40 cycles). Each template was run in triplicate. The sequence for pre-oar-miR-214_3p is GTCATGTGT CTGCCTGTCTACACTTGCTGTGCAGAACATC CGCTCACCTGTACAGCAGGCACAGACAGGC AGTACATGACA, amplified using the tailing method, with primers (miRBase Reference Sequence: MIMAT0000271) ACAGCAGGCACAGACAGGC. The internal control was U6 with the following primers (NCBI Reference Sequence: NR_138085.1): F: TGGAACGCTTCACGAATTTGCG, R: GGAACGATACAGAGAAATTAGC. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Northern blot nucleic acid hybridisation

Agarose (0.2 g) was dissolved in 12.4 ml of DEPC-treated H_2O and heated to melt. Subsequently, 4 ml of 5× formaldehyde gel electrophoresis buffer and 3.6 ml of 37% formaldehyde were added and thoroughly mixed to prepare the gel. After the gel solidified, pre-electrophoresis was performed in 1× formaldehyde gel electrophoresis buffer for 5 minutes. Next, 4.5 µl of total RNA (approximately 20–30 µg) was added to a mixture of 4 µl of 5× formaldehyde gel electrophoresis buffer, 3.6 µl of 37% formaldehyde, and 10 µl of formamide. The mixture was incubated at 65 °C for 15 min and ice-cooled for 5 minutes. Then, 1 µl of nucleic acid stain (1 µg/µl) and 2 µl of loading buffer were added to the mixture, which was then subjected to electrophoresis at 50 V. The RNA was transferred from the denaturing gel to a nitrocellulose membrane. Probe labelling was performed using the Prime-a-Gene Labelling System (Promega) as follows: 25 ng of template DNA was denatured at 95–100 °C for 5 min and cooled on ice for 5 min; dGTP (1 µl), dATP (1 µl), dTTP (1 µl), dNTPmix (2 µl), BSA (2 µl; 10 mg/ml), 5× buffer (10 µl), Klenow enzyme (1 µl), α-32P-dCTP (5 µl), and ddH_2O were added to make a total volume of 50 µl; the mixture was incubated at room temperature for 1 hour. The membrane was placed in a hybridisation bottle with the reverse side facing inward and pre-hybridised with 5 ml of the pre-hybridisation solution at 42 °C for 3 hours. The denatured probe was added to the pre-hybridisation solution and hybridised at 42 °C for 16 hours. After removing the hybridisation solution, the membrane was washed with 2 × SSC/0.1% SDS at room temperature for 15 min and 0.2 × SSC/0.1% SDS at 55 °C for 15 min (repeated twice). Exposure was then performed using the following probes:

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U6 probe: biotin-labelled CGTTCCAATTTTA GTATATGTGCTGCCGAAGCGAGCAC; pre-oar-miR-214_3p probe sequence: biotin-labelled CTGCCTGTCTGTGCCTGCTGT.

Image-J software was used to evaluate the gene expression by collecting the grayscale values of each band.

FISH experiment

Fresh ovarian tissues and other samples from Liangshan Black Sheep at different oestrus stages (pre-oestrus, oestrus, post-oestrus, and inter-oestrus) were collected, washed with phosphate-buffered saline (PBS), and fixed in 2% formaldehyde at 4 °C for 24 hours. After two PBS washes, the tissues were embedded in paraffin and sectioned. Deparaffinisation was performed with xylene, followed by hydration using a gradient alcohol. The sections were incubated with 3% H_2O_2 at 37 °C for 10 min and washed with PBS for 2 min, with the process repeated three times.

In situ hybridisation was performed, followed by xylene transparency and neutral resin mounting. The samples were observed under a microscope, and each sample was analysed in triplicate. The pre-oar-miR-214_3p primer was FAM-ACA GCAGGCACAGACAGGCAGU.

Target gene validation

The mTOR and Sema4d wild-type and mutant vectors were constructed, and healthy Liangshan Black sheep ovarian granulosa cells were cultured. The cells were seeded in six-well plates. When the cell density reached 70–90%, the constructed mimic NC, mimics, inhibitor NC, and inhibitor were transfected into the ovarian granulosa cells. After 48 h, RT-qPCR was used to analyse the target gene expression in each group, including the control, mimic NC, mimics, inhibitor NC, and inhibitor groups. The primer sequences for *mTOR* and *Sema4D* are as follows. *mTOR* (NCBI Reference Sequence: NM_001145455.1): F: CACAAGTCGAGCTGCTCATC, R: CTGTG CCTCCAGTTACCAGA; *Sema4D* (NCBI Reference Sequence: XM_060409035.1): F: ACTTCGAGCCTATCCAGAGC, R: AGTTGGAA CCGGCATAGACA.

ELISA test

The culture supernatants from the mimic NC, mimics, inhibitor NC, and inhibitor groups were collected after the intervention. The samples were centrifuged at 2 500 × *g* for 5 min, and the supernatants were collected. Oestrogen concentrations were measured following the instructions on the sheep oestrogen ELISA kit. After the reaction, the optical density (OD) values of each well were determined, and the standard curve and equation were calculated using the four-parameter fitting curve in ELISACalc software to compute the content in each well.

Data analysis

Statistical analysis was performed using SPSS Statistics v21 (IMB, USA). Additionally, *t*-tests and one-way analysis of variance were used for pairwise comparisons between groups and multiple group comparisons, respectively. A *P*-value of <0.05 was considered statistically significant. Graphs were plotted using Origin v8 software (OriginLab, USA).

RESULTS

RT-qPCR

We analysed the gene expression pattern of *oar-miR-214_3p* in the ovarian and uterine tissues of Liangshan Black sheep during the proestrus, oestrus, metoestrus, and dioestrus phases using RT-qPCR. *Oar-miR-214_3p* expression in the ovaries and uterus varied significantly across the different stages (Figure 1), with the highest levels observed during oestrus ($P < 0.05$).

Northern blotting

Oar-miR-214_3p expression in the ovarian and uterine tissues of Liangshan Black sheep during the proestrus, oestrus, metoestrus, and dioestrus periods was analysed using northern blotting. *Oar-miR-214_3p* was expressed in the ovarian and uterine tissues, with significant differences in expression at different stages (Figure 2). Gene expression was significantly increased during proestrus and oestrus

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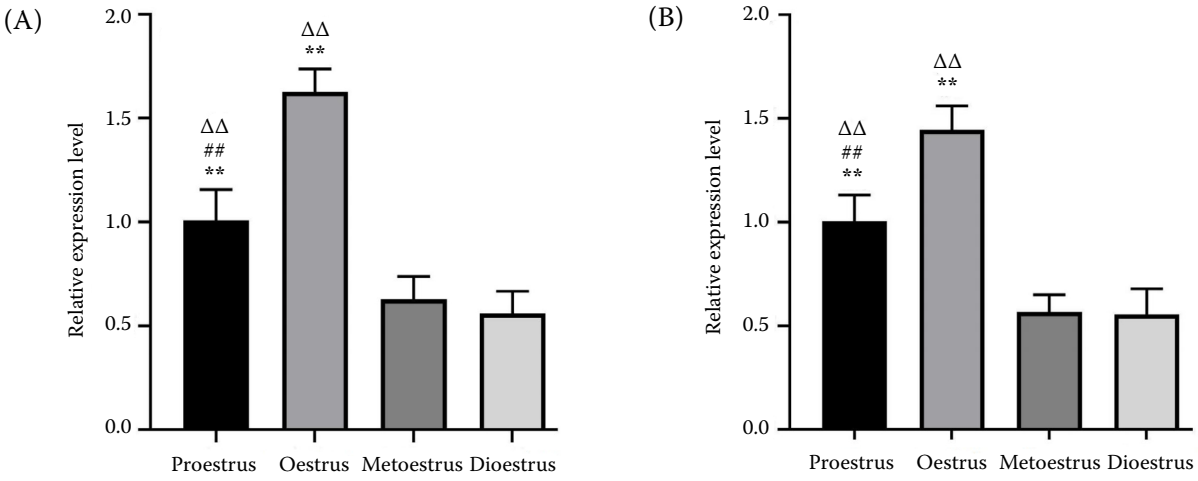

Figure 1. *Oar-miR-214_3p* expression in the ovary (A) and uterus (B) at different stages

**Indicates a significant increase during oestrus and proestrus compared with metoestrus, with $P < 0.05$; ##Indicates a significant decrease during proestrus compared with oestrus, with $P < 0.05$; ΔΔIndicates a significant increase during oestrus and proestrus compared with dioestrus, with $P < 0.05$

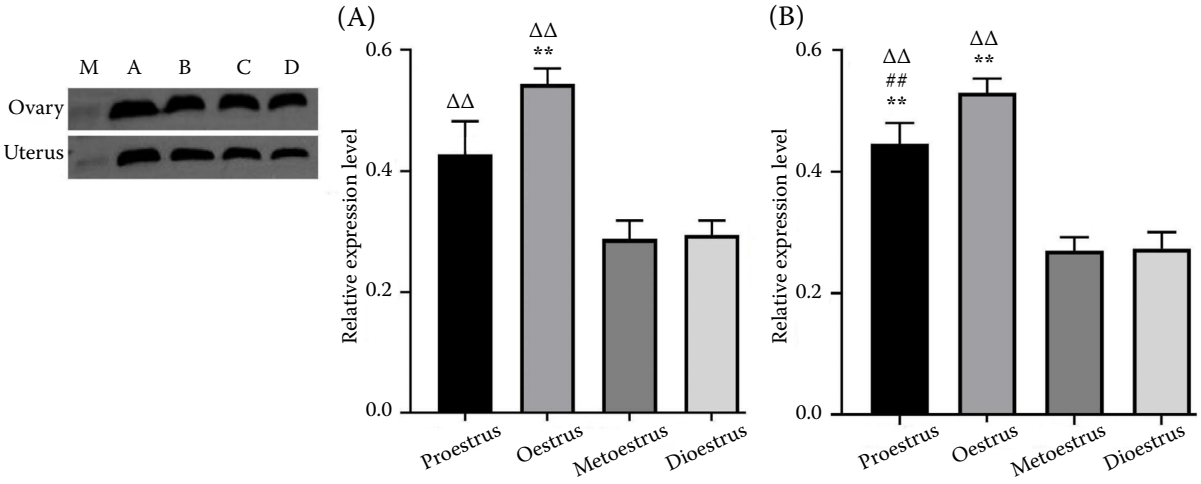

Figure 2. *Oar-miR-214_3p* expression in the ovary and uterus at different stages

In the top-left corner is the NB band diagram, with the grayscale calculation graph on the right; Proestrus, oestrus, dioestrus, and metoestrus, respectively; (A) The ovary, and (B) the uterus; M indicates the marker

**Indicates a significant increase during oestrus and proestrus compared with metoestrus, with $P < 0.05$; ##Indicates a significant decrease during proestrus compared with oestrus, with $P < 0.05$; ΔΔIndicates a significant increase during oestrus and proestrus compared with dioestrus, with $P < 0.05$

($P < 0.05$), with significantly higher expression during oestrus than proestrus ($P < 0.05$). The expression trend was consistent with the RT-qPCR results.

FISH experiment

FISH experiments were conducted on the ovaries of Liangshan Black sheep during proestrus, oestrus, metoestrus, and dioestrus. Oligonucleotide probes for *oar-miR-214_3p* modified with nucleic acids were used for the RNA FISH analyses. Biotinylated conjugated anti-DIG antibodies were used to detect RNA signals, which were amplified with SABC-FITC. *Oar-miR-214_3p* was localised in the cytoplasm (Figure 3).

Based on the fluorescence intensity, *oar-miR-214_3p* expression was upregulated during proestrus and oestrus ($P < 0.05$), with significantly higher expression during oestrus than proestrus ($P < 0.05$). The expression trend was consistent with the RT-qPCR and northern blot results.

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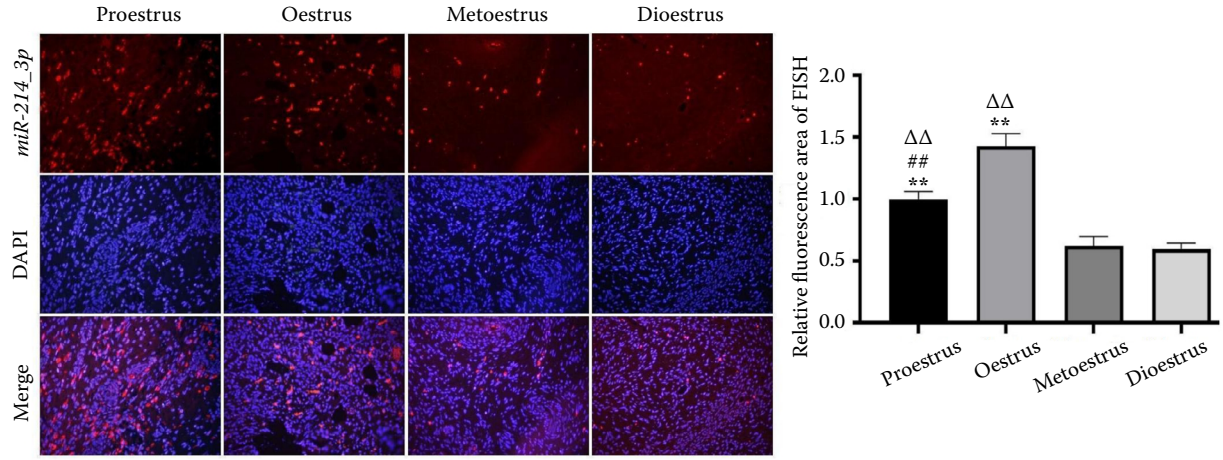

Figure 3. *Oar-miR-214_3p* expression pattern in the ovary at different stages and its localisation in tissue cells
The red fluorescence indicates the location and expression intensity of the probe, while the blue fluorescence represents the nuclei of all cells stained with 4',6-diamidino-2-fenylindol (DAPI)
**Indicates a significant increase during oestrus compared with metoestrus ($P < 0.05$); ##Indicates a significant decrease during proestrus compared with oestrus ($P < 0.05$); ΔΔIndicates a significant increase during oestrus and proestrus compared with dioestrus and metoestrus ($P < 0.05$); Magnification: 20 × 10

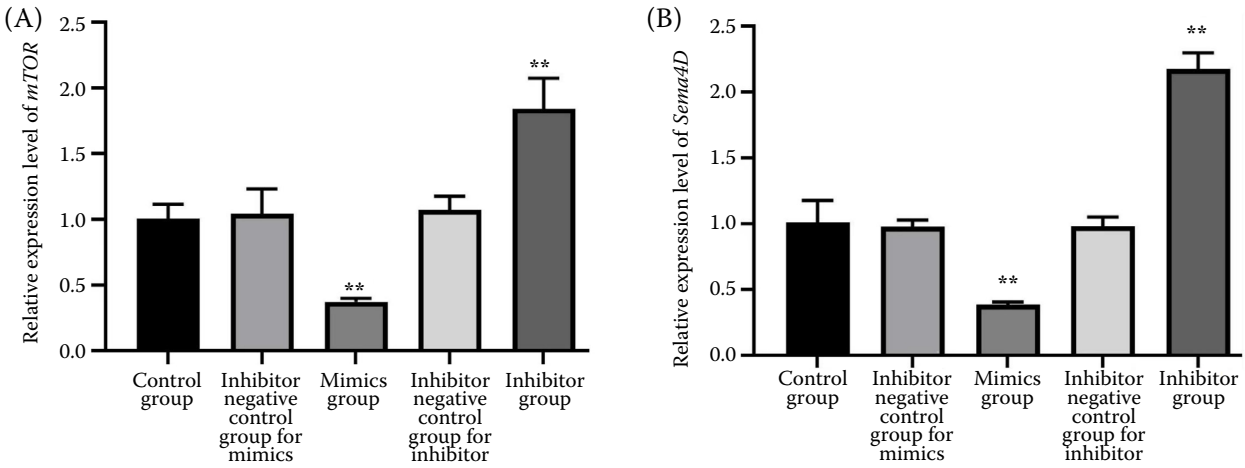

Figure 4. Verification of target genes using a dual luciferase reporter system
(A) Represents the expression level of *mTOR*; (B) Represents the expression level of *Sema4D*
**Compared with the control group, the expression of the target gene in the inhibitor group was significantly increased ($P < 0.05$), while that in the mimics group was significantly decreased ($P < 0.05$)

Dual-luciferase activity assay

Wild-type and mutant vectors for mTOR and Sema4d were constructed, and after 48 h of intervention in granulosa cell culture, RT-qPCR was used to measure mTOR and Sema4D expression. The mimic groups inhibited the expression of both target genes ($P < 0.05$), while the inhibitor groups promoted the expression of the target genes ($P < 0.05$; Figure 4). The expression levels of the target genes in the control and NC groups were relatively stable, with no significant differences. The dual-luciferase reporter system confirmed that *mTOR* and *Sema4D* are *oar-miR-214_3p* target genes.

ELISA experiment

After intervention, the culture supernatants from each well were collected, and the oestrogen concentrations were measured using ELISA. The mimic groups promoted oestrogen secretion ($P < 0.05$),

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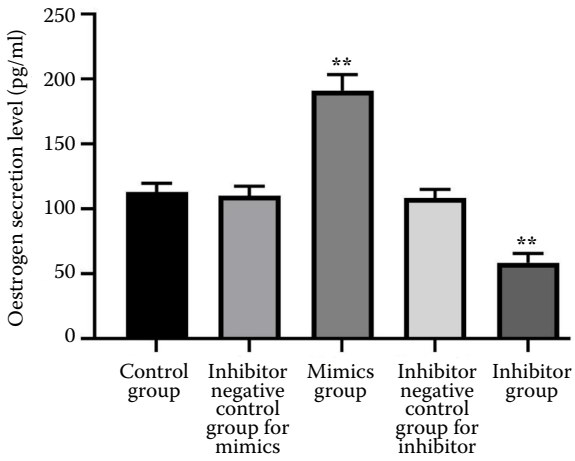

Figure 5. Regulatory effect of *oar-miR-214_3p* on oestrogen secretion

**Compared with the control group, the secretion of estrogen in the inhibitor group was significantly increased ($P < 0.05$), whereas that in the mimics group was significantly decreased ($P < 0.05$)

while the inhibitor group inhibited oestrogen secretion ($P < 0.05$; Figure 5). The oestrogen expression levels in the control and NC groups were relatively stable, with no significant differences. The secretion trend was consistent with the expression trend of the target genes, indicating that *oar-miR-214_3p* participates in the regulation of oestrogen secretion.

DISCUSSION

Animal reproductive physiology is a complex biological process involving the generation, differentiation, and migration of reproductive cells and the growth and development of reproductive organs. Studying animal reproductive physiology provides a better understanding of the biological and theoretical foundations underlying animal reproductive behaviour, thereby offering scientific support for improving reproductive performance and diagnosing and treating reproductive diseases. The Liangshan Black Sheep, also known as the Black Elf, is a single-lamb breed that was listed among China's "Top Ten Excellent Livestock and Poultry Genetic Resources" by the Ministry of Agriculture and Rural Affairs on November 23, 2021. On May 9, 2024, the breed was ranked among the top 500 brands of national geographical indication agricultural products by the China Brand Value 500 Evaluation Committee. This breed, developed by the Yi people in areas such as Butuo County of Liangshan Yi Autonomous Prefecture, is a high-quality genetic resource formed through long-term selective breeding based on the region's unique natural environment and human preferences. It is genetically stable, has tender meat, a mild odour, and strong adaptability, and exhibits tolerance to coarse feed. Furthermore, it is a dual-purpose sheep breed that is primarily relied upon by the Yi people. However, it suffers from low reproductive efficiency and underutilisation. Investigating the reproductive physiology of Liangshan Black Sheep and its regulatory mechanisms and developing Liangshan Black Sheep with a high reproductive rate is crucial for improving livestock farming in Liangshan Prefecture and enhancing the Liangshan Black Sheep brand.

MiR-214 consists of four components, namely *miR-199_5p*, *miR-214_3p*, *miR-199_3p*, and *miR-214_5p* (Su et al. 2024). Its sequence is approximately 22 nucleotides long, and the precursor sequence is processed into mature miR-214 (UGCCUGUCUACACUUGCUGUGC) after transcription and cleavage. MiR-214 is located within an intron of the dynamin-3 gene, while miR-199a is found approximately 6 kb away from miR-214. The miR-199a/miR-214 cluster usually participates in the regulation of the same response. Increasing evidence suggests that miR-214 plays a role in post-transcriptional gene silencing to regulate various biological processes, including cell proliferation, differentiation, migration, apoptosis, body development, and disease progression (Zhong et al. 2023; Trevelyan et al. 2024). An RT-qPCR experiment was performed on Liangshan Black Sheep to assess the *oar-miR-214_3p* expression pattern in the ovarian and uterine tissues during different stages of the oestrous cycle (proestrus, oestrus, metoestrus, and dioestrus phases). The data indicated that *oar-miR-214_3p* presented significant differences in expression across different stages, with significantly higher expression during the pre-oestrus and oestrus stages ($P < 0.05$). Moreover, significantly higher expression was observed during oestrus than proestrus ($P < 0.05$). Additionally, northern blotting of the samples confirmed the *oar-miR-214_3p* expression trend, which was consistent with the RT-qPCR results. During metoestrus, and dioestrus, follicular activity in the ovary was suppressed, while during proestrus and oestrus, oestrogen secretion increased and follicular ac-

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tivity was stimulated. Notably, the dominant follicle expels the egg toward the end of the oestrus. The *oar-miR-214_3p* expression trend aligns with the physiological activities that occur during the oestrous cycle in sheep, suggesting that its expression is related to the oestrous cycle and other physiological processes. Although studies on the regulation of sheep reproductive physiology by *oar-miR-214_3p* are limited, studies on other miRNAs have been reported. For example, Miao et al. (2018) revealed that *miR-150* is differentially expressed in the ovarian tissues of Turpan Black Sheep during the follicular and luteal phases. Sun et al. (2024) demonstrated that *miR-133a-3p* inhibits sperm fertilisation and embryo development by targeting the *circMYH9*/*miR-133a_3p*/*CXCR4* axis, thus providing new insights into the molecular mechanisms controlling sperm function and early embryo development. Tian et al. (2025) confirmed that *gga-miR-6634_5p* regulates the proliferation of granulosa cells and steroid hormone secretion in hens by targeting *MMP16*, providing a theoretical basis for understanding miRNA function in the ovarian development of egg-laying chickens. Wang et al. (2023) found that *miR-449a* is highly expressed in the pituitary while *miR-449b* is highly expressed in the ovary, with their target gene numbers being 299 and 23, respectively, suggesting their involvement in the reproductive regulation of Hu Sheep. Xu et al. (2023) also showed that *miR-214_3p* regulates the differentiation of germ cells by influencing the cell cycle through the regulation of cell cycle-related genes. Thus, our finding that *oar-miR-214_3p* participates in regulating animal reproductive physiology is consistent with these previous findings.

RNA FISH experiments conducted on the ovaries of Liangshan Black Sheep during proestrus, oestrus, metoestrus, and dioestrus included oligonucleotide probes modified for *oar-miR-214_3p*, with the biotin-conjugated anti-DIG antibody used to detect RNA signals. Signal amplification was performed with SABC-FITC, which revealed that *oar-miR-214_3p* localised to the cytoplasm. *Oar-miR-214_3p* expression was upregulated during pre-oestrus and oestrus ($P < 0.05$). Wild-type and mutant vector constructs of mTOR and Sema4D were transfected into the granulosa cells of Liangshan Black Sheep after 48 h of culture. RT-qPCR analysis of mTOR and Sema4D expression revealed that the mimic treatment groups inhibited the expression of both target genes while the inhibitor treatment groups promoted their expression. The control and NC groups showed stable target gene expression with no significant differences. Dual-luciferase reporter assays confirmed that *mTOR* and *Sema4D* were *oar-miR-214_3p* target genes. *mTOR*, a 289 kDa serine/threonine kinase, belongs to the phosphoinositide 3-kinase (PI3K) related kinase family. The mTOR pathway plays a central role in controlling cell growth and proliferation, integrating signals from nutrients, energy status, and growth factors to regulate various life processes, including cell growth, proliferation, autophagy, and metabolism (Liu et al. 2023; Qian et al. 2023; Shi et al. 2024). SEMA4D, also known as CD100, is a signalling protein in the nervous system and a member of the class 4 SEMA family (Alto and Terman 2017; Iragavarapu-Charylu et al. 2020). SEMA4D is a transmembrane homodimer glycoprotein of 150 kDa that contains an N-terminal signal sequence, a C2-type Ig domain, hydrophobic transmembrane region, and cytoplasmic tail. It plays a significant regulatory role in cell adhesion, proliferation, migration, morphological changes, immune response, and angiogenesis (Alto and Terman 2017; Al Turkestani et al. 2023; Xuan et al. 2024). In 2023, Liu et al. (2023) showed that Prohibitin 2 binds to ERβ and targets the mTOR pathway to induce autophagy in porcine granulosa cells. Wang et al. (2024) found that in Zhedong white geese, during nesting, follicular atrophy and apoptosis occurred in the ovaries, and significant changes were observed in serum reproductive hormone levels. *COL3A1*, *COL1A2*, *GRIA1*, *RNF152*, *miR-192*, and *miR-194* were identified as significant candidate genes for regulating nesting behaviour, with the mTOR signalling pathway playing a crucial role. Chen et al. (2022) showed that reduced *SEMA4D* expression in the endometrium impairs trophoblast cell invasion and migration via the Met/PI3K/Akt pathway, providing insights into recurrent implantation failure. Recent studies further reveal that in preeclamptic placental tissues, *SEMA4D* and its receptor, Plexin-B1, are significantly reduced, with levels lower than those of the normal control group. *SEMA4D* enhances the binding of Plexin-B1 to the tyrosine kinase receptor Met, promoting Met/Erk phosphorylation and increasing trophoblast cell invasion, which aids placental formation (Li et al. 2018). These findings suggest that *mTOR* and *SEMA4D* are involved in regulating animal reproductive physiology. Dual-luciferase reporter

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assays confirmed that *mTOR* and *Sema4D* are target genes of *oar-miR-214_3p*, indicating its role in regulating the oestrous cycle in sheep.

In this study, wild-type and mutant vector constructs were transfected into granulosa cells, and oestrogen levels in the culture medium were detected using ELISA. Data analysis indicated that oestrogen secretion was promoted and suppressed by the mimic and inhibitor treatment groups, respectively. The control and NC groups exhibited stable oestrogen expression levels with no significant differences. Studies suggest that miR-214 regulates steroidogenesis in rat granulosa cells by targeting the low-density lipoprotein receptor gene, playing a crucial role in steroid synthesis and secretion (Tian et al. 2018). Shi et al. (2020) showed that *miR-214_3p* promotes granulosa cell proliferation by targeting *Mfn2* and inhibits oestrogen synthesis by targeting *NR5A1/SF-1*, playing an essential role in initiating puberty and regulating the oestrous cycle.

According to the experimental results, which are supported by previous studies, *oar-miR-214_3p* participates in the regulation of oestrogen secretion in sheep ovarian granulosa cells. Martinez et al. (2018) found that *hsa-miR-214* controls oocyte or follicular development by targeting the PTEN-PI3K-Akt pathway. Further in-depth study on this mechanism is crucial for understanding the role of miRNA in ovulation regulation in animals, with potential applications in timed breeding for livestock. Zeng et al. (2020) demonstrated that 2,5-hexanedione inhibits PI3K signalling by upregulating *miR-214_3p*, causing the suppression of primordial follicle development in neonatal ovaries, confirming that miR-214 is involved in follicular development. In this study, *oar-miR-214_3p* expression in ovarian and uterine tissues was significantly elevated during oestrus and proestrus phases. Similarly, it was confirmed to participate in oestrogen secretion by sheep ovarian granulosa cells. Oestrogen secretion peaks before ovulation, indicating that *oar-miR-214_3p* plays a role in follicular development and ovulation, which is consistent with previous study findings (Shi et al. 2020; Zeng et al. 2020; Hou et al. 2023; Xu et al. 2023).

However, the specific pathways through which *oar-miR-214_3p* regulates ovarian follicular development, ovulation, and oestrogen secretion in Liangshan Black Sheep require further investigation.

CONCLUSION

The *oar-miR-214_3p* gene plays a crucial role in regulating ovarian follicle development, ovulation, oestrogen secretion, and the oestrous cycle in sheep via its target genes *mTOR* and *Sema4D*. This study highlights the potential of *oar-miR-214_3p* as a target for improving reproductive efficiency in sheep through molecular breeding techniques.

Future research should focus on elucidating the specific pathways by which *oar-miR-214_3p* regulates these processes.

Conflict of interest

The authors declare no conflict of interest.

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