

RESEARCH ARTICLE

MicroRNA-200c Mediates the Mechanism of MAPK8 Gene Regulating Follicular Development in Sheep ^[1]

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Abstract

Most sheep breeds are seasonal estrus and single birth animals. In order to improve pastoral area economy and lambing rate, In order to improve pastoral area economy and lambing rate, and It is very important to study the key genes affecting sheep reproductive traits at the molecular level. On the basis of early verification of the relationship between miR-200c and MAPK8 gene target. In this experiment, after over-expression of miR-200c in ovarian granulosa cells, the expression of mitogen-activated protein kinase 8 (MAPK8), frizzled class receptor 3 (FZD3), G protein subunit alphaq (GNAQ), jun proto-oncogene (JUN), protein kinase C beta (PRKCB) in estrus-related pathway genes was detected by qRT-PCR, and the expression of estradio (E_2) and progesterone (P_4) in reproductive stimulation was detected by ELISA. The relationship between MAPK8 gene and follicular ovulation was analyzed. The results showed that up-regulated corpus follicular mRNA expressions of FZD3, GNAQ ($P<0.01$), and down-regulated mRNA expressions of MAPK8, PRKCB ($P<0.05$) and JUN ($P<0.01$). The secretion of E_2 first increased and then decreased with the passage of time, while the secretion of P_4 first increased and then returned to the normal level with the passage of time, which was consistent with the development of follicles in mammalian estrus. In summary, miR-200c-mediated MAPK8 gene has a strong promoting effect on E_2 secretion and a certain regulating effect on P_4 , suggesting that miR-200c-mediated target gene MAPK8 plays a regulating role in follicular development.

Keywords: Kazakh sheep, miR-200c, MAPK8, Ovulatory number

MicroRNA-200c Koyunda MAPK8 Gen Düzenleyici Foliküler Gelişim Mekanizmasına Aracılık Eder

Öz

Koyun ırklarının çoğu mevsimsel östrus gösteren ve tek doğum yapan hayvanlardır. Kırsal alan ekonomisini iyileştirmek ve kuzulama oranını artırmak için, miR-200c ve MAPK8 gen hedefleri arasındaki ilişkinin erken doğrulanması temelinde koyun üreme özelliklerini etkileyen önemli genleri moleküler düzeyde incelemek oldukça önemlidir. Bu çalışmada, yumurtalık granüloza hücrelerinde miR-200c'nin aşırı ekspresyondan sonra, östrusla ilişkili yolak genlerinden Mitojen Aktiveli Protein Kinaz 8 (MAPK8), Frizzled sınıf reseptör 3 (FZD3), G Protein Altünite Alfa Q (GNAQ), Jun Protoonkogen (JUN), Protein Kinaz C Beta (PRKCB) ekspresyonu QT-PCR ile ve üreme stimülasyonunda östradiol (E_2) ve progesteron (P_4) ekspresyonu ELISA ile saptandı. MAPK8 geni ile foliküler ovulasyon arasındaki ilişki incelendi. Sonuçlar, FZD3, GNAQ'nın korpus foliküler mRNA ekspresyonlarının arttığını ($P<0.01$) ve MAPK8, PRKCB ($P<0.05$) ve JUN ($P<0.01$)'un mRNA ekspresyonlarının ise azaldığını gösterdi. Memeli östrusundaki folikül gelişim ile uyumlu olarak E_2 salgısı başlangıçta arttı sonra zamanla azalırken, P_4 salgısı başlangıçta arttı ve daha sonra zamanla normal seviyeye döndü. Özetle, miR-200c aracılı MAPK8 geninin E_2 salgılanması üzerinde güçlü bir teşvik edici etkisi ve P_4 üzerinde belirli bir düzenleyici etkisi vardır, bu da miR-200c aracılı hedef gen MAPK8'in foliküler gelişimde düzenleyici bir rol oynadığını düşündürmektedir.

Anahtar sözcükler: Kazak koyunu, miR-200c, MAPK8, Yumurtlama sayısı

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INTRODUCTION

The breeding characteristics of sheep are closely related to the production cost and efficiency of breeding industry. However, increasing the number of lambs born is the main measure to improve the economic benefits of sheep field. In order to improve the utilization efficiency, increasing twin or multiple birth rate, of Jining grey goat, more study can be done on molecular genetics^[1]. It is very important to study the molecular genetic mechanism of non-degenerate multiple fetuses in sheep. In the endocrine reproductive system, pregnancy and lactation are regulated by reproductive hormones since the endocrine hormones and reproduction are closely related^[2]. The oogenesis and folliculogenesis are closely linked and occur simultaneously in the growing ovarian follicles. Granulosa cells are highly complex and depend on many factors, including intercellular communication. Studies have shown that the hypothalamic-pituitary-gonadal axis (HPG) is related to multiple births, in which hormone regulation is particularly important. Released by the hypothalamus, gonadotropin-releasing hormone (GnRH) to stimulate the pituitary gland synthesis and follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretion^[3], thereby promote follicle maturation and ovulation, so reach the role of adjusting the estrus cycle^[4]. Follicle development involves multiple regulatory factors and complex regulatory networks formed by signaling pathways, such as FSH, LH and E₂, and prostaglandin (PGs), which play an important role in regulating the estrus cycle^[5]. There is also new evidence in recent years that the transforming growth factor- β (TGF- β) superfamily plays an important role in early follicular development, granulosa cell proliferation, and differentiation^[6]. These factors act on the granulosa cells on the follicular membrane and regulate the proliferation, differentiation and apoptosis of follicular granulosa cells. Studies have shown that estrogen can negatively regulate the secretion of GnRH and LH by upstream hypothalamic-pituitary tissues, its signal transduction is achieved by activating the MAPK signal transduction system. In addition, PRKCB is also found to indirectly affect MAPK cascade and follicular ovulation^[7-9]. Further studies found that plasma concentrations of P₄ and E₂ in high-fecundity sheep were higher than that of low-fecundity sheep, and were significantly positively correlated with ovulation^[10]. A study showed that FSH, LH and 17 β -E₂ were secreted for many times under their synergism, and LH receptors were produced in follicular granulosa cells. Under the action of LH, more follicles matured and ovulated, which was the main cause of multiple fetters^[11,12].

The role and transcription level of miRNA in transcriptional regulation of gene expression have attracted extensive attention from scientists, who have found that miRNA can regulate follicular development in sheep. For example, miR-200 is expressed in tumors and is highly expressed in bladder and cervical cancers, related to the proliferation of

tumor cells^[13,14] suggested that the expression of miR-200c in the serum of heavy calcium carbonate (HCC) patients was down-regulated, which was associated with tumor size and tumor node metastasis (TNM) staging. At the same time, Zhang et al.^[15] found that miR-200c can specifically inhibit the activity of Rho E 3'-UTR reporter gene, which suggested that miR-200c may have an inhibitory effect on Rho E and affect the occurrence and development of gastric cancer. In addition, Kuang et al.^[16] found that miR-200c contain similar oncogenic and anti-tumor genes in the development and progression of epithelial ovarian cancer. At present, the research on follicular development regulation is still at the primary stage. However, the miR-200c play a very important role in regulating follicular development, but its role in follicular development has not been proven.

In the early stage, we have predicted the differential expression of miRNA using Targetscan and RNAhybrid software, analyzed the target genes through GO enrichment analysis and DAVID's KEGG pathway analysis, screened out the differentially expressed gene miR-200c, and confirmed the negative regulatory relationship between miR-200c and the target gene MAPK8. In this study, the effects of miR-200c on ovarian granulocyte cells and some estrus-related genes were investigated to determine the role of MAPK8 gene in follicular development, providing a theoretical basis for the study of seasonal estrus and reproductive performance of sheep.

MATERIAL AND METHODS

Sample Collection

Kazakh ewes were raised in the animal laboratory of Shihezi University animal experiment station. Upon confirmation of their reproductive status, these ewes were injected with sodium pentobarbital. After slaughter, the uterus was taken out and the ovary in follicular phase was screened out, and placed in a sampling bucket containing 1% PBS, and transferred to a laboratory ultra-clean station for ovarian granulosa cell culture within 30 min.

Culture and Identification of Cells

Ovaries were recovered at slaughter and transported to the laboratory at 38°C in 1% PBS within 30 min. The ovaries of each animal were placed in 1% PBS supplemented with fetal bovine serum (FBS, Sigma-Aldrich Co, St.Louis, MO, USA). After that, single preovulatory large follicles, with an estimated diameter greater than 5 mm, were opened into a sterile Petri dish by puncturing with a 5 mL syringe and 20-G needle. The culture medium consisted of Dulbecco's Modified Eagle medium F12 (DMEM-F12, Sigma-Aldrich, USA), 10% fetal calf serum (FCS; increased concentration required to maintain adequate cell viability in culture) (PAA, Linz, Austria), 1% penicillin and streptomycin (Invitrogen, USA). The cells were cultured at 38.5°C under aerobic

conditions (5% CO₂). The medium was changed every 48 h.

Screening of cell growth density is more than 80% of the cells in a petri dish typically increases, at room temperature in 4% paraformaldehyde fixed solution after 15 min. After incubation at room temperature with 3% H₂O₂ for 10 min to eliminate internal peroxidase activity, after incubation with 0.1% tritonx-100 (Sigma-Aldrich, China) at room temperature for 20 min, after blood was discarded, 10% goat serum (Gibco, China) was incubated at room temperature for 1 h without washing. 2 mL monoclonal antibody (Anti-JNK1+JNK2 (T183+Y185 phosphate), the working fluid concentration is 1:200, Shanghai biological engineering co, LTD, China) was added and incubated at 4°C for 12 h. After that, FITC label, diluted 1:200, at 37°C for 1 h, and later with the same amount of DAPI (Sigma-Aldridge, China) dye solution at room temperature for 15 min were applied. Fluorescent anti-quench seal solution (Sigma-Aldrich, China) was added to avoid light. Cell staining was observed under an inverted confocal fluorescence microscope.

Oligonucleotide Transfection of Sheep Ovarian Granulocytes

Cells were transfected with 100 nmol/L of miR-200c mimics. Single-strand oligonucleotides were designed to specifically bind and inhibit endogenous miRNAs. Ovarian granulocytes were inoculated in a 6-well plate and upon reaching to 70-80% confluency, these were transfected using Lipofectamine™ 2000 (sigma-Aldrich, China) according to the manufacturer's instructions. NC is the blank group, miR-200c is the interference group, and miR-200c Inhibitor is the interference control group.

QT-PCR and ELISA were Used to Detect the Effect of miRNA-200c on Estrus Related Genes and Hormones

Total RNA of the cells collected was extracted by the Trizol reagent (Thermo Fisher Scientific, China), cDNA was synthesized by Takara-PrimeScript™ RT reagent Kit. Expression value of related reproductive gene was verified by quantitative Real-time polymerase chain reaction (QT-PCR). The primers used were listed in *Table 1*. The PCR

instrument was ABI PRISM 7500 (Applied Biosystems, Carlsbad, California). This was done using the QuantStudio 7 Flex fluorescent quantitative PCR instrument (Applied Biosystems, USA). PCR amplification was performed in 20 µL of reaction mixture that contained 10 µL SYBR Premix EX Taq II (TAKARA Bio Inc., Dalian, China), 1 µL of each forward and reverse primer, 7 µL RNase-Free ddH₂O, and 2 µL cDNA (200 ng/µL). PCR amplification was performed in triplicate wells using the following conditions: initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec, and 60°C for 30 sec. The dissociation curve was analyzed after amplification. A melting temperature (T_m) peak at T_m±0.8°C on the dissociation curve was used to determine the specificity of PCR amplification. Sheep β-actin gene was selected as housekeeping genes.

Collect groups of 6 h and 24 h after transfection cell culture. Samples were stored separately at -20°C to maintain protein activity and avoided repeated freezing. E₂ and P₄ levels were detected according to the ELISA kit instructions (Ovine E₂ ELIA kit, Ovine P₄ ELISA kit, BLUEGENE, China).

Analysis of Data

Results were analysed using SPSS17.0. 2^{-ΔΔCt} was used to calculate the relative expression level. P<0.05 was considered significantly different, and P<0.01 was accepted highly significantly different.

RESULTS

Ovarian Granulosa Cell Identification

Ovarian granulosa cells were identified after DAPI staining, and the nucleus of the ovarian granulosa cells was seen blue (*Fig. 1-A*), and the cytoplasm was mainly green, and the immune response product of the target gene *MAPK8* was distributed in the cytoplasm, as indicated by the red arrow (*Fig. 1-B*). When A and B were observed in combination, the cytoplasm was fluorescent green and the nucleus was blue (*Fig. 1-C*), the results showed that the transfection was successful and the follow-up experiments could be carried out.

Table 1. Primer information for qRT-PCR

Gene	Primers for QT-PCR	T _m (°C)	Product Length (bp)
FZD3	F: TACCTTCATGCCCAATCTTCTG R: CGAGGATACGGCTCATCACAAAT	60.29	288
GNAQ	F: AGACAATGAGAACCGAATGGAG R: GAAATAGTCAACTAGGTGGGAATACA	58.00	151
JUN (C-Jun)	F: ACGACCTTCTACGACGATGCCCTCA R: ACGAAGCCCTCGGCGAATCCCT	68.25	326
MAPK8	F: AGCAGTGACCAGTGGCTCTCAG R: CAGCCGACGCTTCTAGACTGC	64.21	119
PRKCB	F: GCGTCCTGCTGTATGAGATGTTG R: AGCCACGTTGTGTTCCATGATCG	63.78	96
β-actin	F: TCGTTGTAGAAGGTGTGGT R: AGAGCAAGAGAGGCATCC	55.96	103

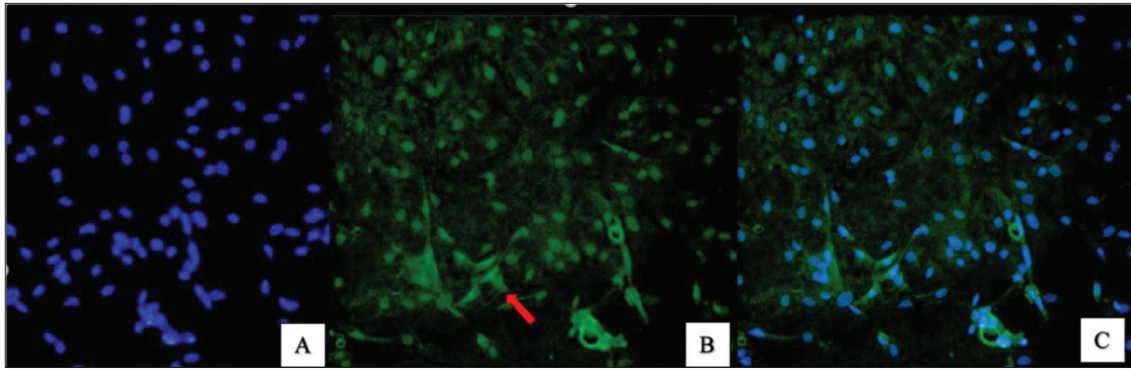


Fig 1. Immunohistochemical identification of cells

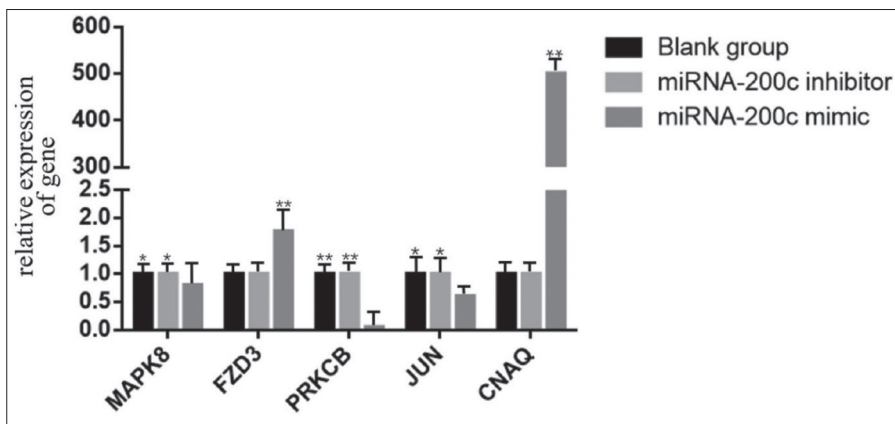
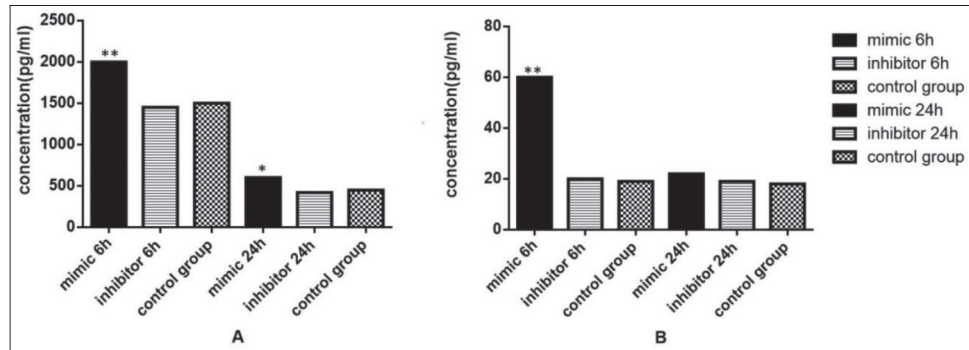


Fig 2. Expression levels of related genes after transfection of miR-200c. The significant results with a P-values lower than 0.01 and 0.05 are given two asterisks (**) and one asterisk (*), respectively

Fig 3. Contents of E₂ and P₄ in ovarian granulosa cell culture medium after transfection for 6 h and 24 h (A is the content of E₂ in ovarian granulosa cell culture medium after transfection for 6 h and 24 h; B is the content of P₄ in ovarian granulosa cell culture medium after transfection for 6 h and 24 h. The significant results with a P-values lower than 0.01 and 0.05 are given two asterisks (**) and one asterisk (*), respectively)



QT-PCR was Used to Detect Changes in Estrus Related Genes

In order to further study the regulation effect of miRNA on the 3'UTR region of target gene MAPK8 and other estration-related genes, mRNA expression levels of target gene MAPK8 and other estration-related genes in ovarian granulocytes of Kazak sheep were detected by QT-PCR. In this study, MAPK8 upstream and downstream genes in estration-related *GnRH* signaling pathway and Wnt signaling pathway were selected for expression verification. After the overexpression of miR-200c (Fig. 2), the expression of MAPK8 gene was significantly down-regulated (P<0.05), and the upstream FZD3 gene was significantly up-regulated (P<0.01).The expression of PRKCB gene was very significantly down-regulated (P<0.01), the

expression of downstream *JUN* gene was significantly down-regulated (P<0.05), and the expression of miR-200b target gene *GNAQ* in our research group was highly significantly up-regulated (P<0.01).

ELISA Detection Results of E₂ and P₄

After transfection for 6 h, E₂ level in the mimic group was significantly higher than that in the inhibitor group and control group (P<0.01). Twenty-four h after transfection, E₂ level in the mimic group was significantly higher than that in the inhibitor group and control group (P<0.05) (Fig. 3-A). The results indicated that E₂ level increased after transfection with miR-200c mimetic.

After transfection for 6 h, the level of P₄ in the mimic group was significantly higher than that in the inhibitor

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group and control group ($P < 0.01$). Twenty-four h after transfection, E_2 level in the mimic group was significantly higher than that in the inhibitor group and control group ($P < 0.05$) (Fig. 3-B). P_4 level increased 6 h after transfection of miR-200c mimetic, but did not change significantly at 24 h after transfection.

The results indicated that miR-200c-mediated MAPK8 gene could promote E_2 secretion and regulate P_4 .

DISCUSSION

Early by our team, miR-200a/b/c was found to have different expressions in hypothalamus, pituitary, ovary, uterus, uterine horn, and fallopian tube. We analyzed the tissue expression profile of miR-200a/b/c in Kazakh sheep. The results suggested that miR-200a/b/c may be involved in the regulation of estrus by regulating different target genes in animals [14,17].

In experiments on sheep hypothalamus cells, it was found that miR-200b could regulate the expression of GnRH by down-regulating GNAQ, thus affecting the estrus of sheep [18]. Studies have shown that follicular ovulation can be affected and follicular development further regulated by interfering with the expression of the PLA2G4D gene (the down-regulated gene of PRKCB) [19]. The results of this experiment showed that after the overexpression of miR-200c, the expression of PRKCB and MAPK8 genes were significantly down-regulated. To sum up, MAPK8, as a down-regulation gene, would feedback and regulate the expression of follicle development-related genes in GnRH synthesis pathway and Wnt signaling pathway. We speculated that MAPK8 was an important gene promoting follicular development. Related studies have shown that binding of C-Jun amino-terminal kinase MAPK8 and transcription factor ATF2 upon phosphorylation of C-Jun amino-terminal kinase MAPK8 results in phosphorylation of the binding region, which binds to AP-1 on the gene promoter to promote cell proliferation and cell differentiation [20]. When the expression of MAPK8 stagnated in the morula stage, a cavity was formed, leading to incomplete oocyte development. In addition, Wang et al. [21] found that the JNK signaling pathway was blocked after MAPK8 was silenced and activated after MAPK8 overexpression, suggesting that MAPK8 may inhibit follicular development. Other studies have shown that up-regulation of FZD3/5 and activation of Wnt signaling pathway can reduce oxidative stress injury of neurons in Alzheimer's disease model in mice and inhibit cell apoptosis [22]. This is consistent with the results of our experiment, with the over-expression of miR-200c and the significantly down-regulated downstream gene JUN.

The researchers also found that FSH and estrogen, combined with increased LH and FSH receptors in follicular granulosa cells, combined with the ovary further stimulated the follicle to produce more estrogen [23]. Similar studies have

shown that the development of sheep reproductive system is positively correlated with E_2 secretion in LH and follicular cells. E_2 increases in early stage and estrogen gradually increases. Total follicular growth was positively correlated with E_2 content in plasma [24]. Nogueira et al. [25] believed that the high content of P_4 and low content of E_2 in the non-breeding season were important factors leading to anorexia in sheep. Instead, reducing P_4 levels and increasing E_2 levels during the non-breeding season may promote estrus, where higher P_4 levels lead to higher levels of pituitary follicle stimulating hormone, which further retards follicular development. The lower the level of P_4 , the lower the release of LH and the weaker the inhibitory effect of pituitary, leading to increased FSH, which further stimulates follicular development.

In our studies, enzyme-linked immunosorbent assay was used to detect the content of reproductive hormones (E_2 and P_4) at different stages after the overexpression of miR-200c. The results showed that the levels of E_2 and P_4 increased after transfection of miR-200c mimetic. Especially, P_4 level increased 6 h after transfection of miR-200c mimetic, but did not change significantly 24 h after transfection, which was consistent with follicular development in estrus of mammals. In short, MAPK8 ACTS as a down-regulated gene, regulating the expression of genes related to follicular development in GnRH synthesis pathway and Wnt signaling pathway. It can be concluded that MAPK8 gene mediated by miR-200c has a strong promoting effect on E_2 secretion and a certain regulatory effect on P_4 .

In conclusion, we found that MAPK8 gene was significantly down-regulated after miR-200c overexpressed in ovarian granulosa cells, which indirectly affected the expression of other genes related to follicular development and comprehensively regulated follicular development. Moreover, MAPK8 gene mediated by miR-200c has a strong role in promoting E_2 secretion, and E_2 has a certain regulatory effect on P_4 , thus promoting follicular ovulation. We can conclude that MAPK8, a target gene mediated by miR-200c, plays an important role in follicular development, providing a theoretical basis for improving the yield and number of young sheep.

AUTHOR CONTRIBUTIONS

NY is the executor of the experimental design and experimental research of this study. NY and JY finished data analysis and writing the first draft of the paper. YH and ZM participated in experimental design and analysis of experimental results. ZZ is the designer and leader of the project, guiding experimental design, data analysis, thesis writing and revision. All the authors have read and agreed to the final text.

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