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BMP15 regulates AMH expression via the p38 MAPK pathway in granulosa cells from goat

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ABSTRACT

Anti-Mullerian hormone (AMH), a member of the TGF-β superfamily, is produced by granulosa cells (GCs) of preantral and small antral follicles and plays a role in regulating the recruitment of primordial follicles and the FSH-dependent development of follicles. However, the regulation of AMH expression in follicles remains poorly understood. The objectives of this study were to determine the following: 1. the association between bone morphogenetic protein 15 (BMP15) and AMH; 2. whether BMP15 can regulate the expression of AMH by inhibiting the p38 MAPK pathway; and 3. whether SRY-related HMG box 9 (SOX9), a transcription factor for *AMH*, is involved in the regulation of AMH expression by BMP15. In this study, an inhibitor of p38 MAPK and an siRNA specific for *p38 MAPK* were used to prevent the function of the p38 MAPK signaling pathway. Then, AMH mRNA expression and AMH secretion were detected in goat GCs using an RT-PCR assay and ELISA, respectively, after treatment with BMP15. The results indicated that BMP15 up-regulates the transcription of *AMH* and that the inhibition of p38 MAPK decreases the BMP15-induced expression of AMH and SOX9, suggesting that BMP15 up-regulates the expression of AMH via the p38 MAPK signaling pathway, and this process involves the *SOX9* transcription factor.

1. Introduction

Anti-Mullerian hormone (AMH), also called Mullerian-inhibiting substance (MIS), is a member of the TGF-β superfamily and is well known for its role in Mullerian duct regression during male sexual differentiation [1]. In females, AMH is mainly expressed in the granulosa cells (GCs) of preantral and small antral follicles [2–5], and AMH has been shown to inhibit the recruitment of primordial follicles. The expression levels of AMH vary during the different developmental stages of follicles. AMH expression begins in primary follicles and reaches a maximal level in small antral follicles [6]. Reports have shown that the serum/plasma AMH level can be a reliable marker of ovarian reserves and the ovarian response to gonadotropin-based stimulatory treatments in humans, bovines and goats [7–10].

The regulation of AMH expression in follicles remains poorly understood. Several factors may affect the expression of AMH. For example, Joelle Taieb et al. [11] recently determined that *AMH* transcription in granulosa cells is enhanced by the FSH- and cAMP-mediated activation of the AMH promoter through protein kinase A (PKA)/p38-dependent signaling pathways. In addition, a stimulating effect of the bone morphogenetic proteins (BMPs) on AMH expression has been proven in human, hen, bovine, and ovine GCs *in vitro* [12–16]. Because of the importance of the BMP system in regulating the reproductive process, additional studies have focused on the mechanism underlying the contribution of BMPs to ovarian function. BMPs also belong to the TGF-β superfamily and play important roles as autocrine and/or paracrine factors in regulating the development of follicles [17]. In particular, BMP15, which is produced by oocytes, has been shown to be involved in the regulation of follicular [18,19] and cumulus cell function [20–23], and BMP15 is found in the oocytes of all types of follicles and in the GCs from primary follicles onward [24]. In addition, BMP15 mutations are related to reproductive activity. As components of the TGF-β superfamily, small mothers against decapentaplegic (SMAD) proteins, when phosphorylated, have been found to be related to BMP function [17]. Studies have shown that BMP15 and BMP4 activate the SMAD1/5/8 signaling pathway by inducing the phosphorylation of the SMAD proteins, which are then transferred to the

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nucleus to activate the transcription of target genes [26,27]. Recently, several reports have shown that BMPs may regulate AMH via the SMAD signaling pathway [16,25]. The p38 MAPK protein is expressed in oocytes and GCs and regulates oocyte maturation and fertilization [28]. And the phosphorylation of the p38 MAPK pathway plays an important role in the regulation of follicular growth and development. It has been found that the second messenger cAMP of LH and FSH can stimulate the transcription of AMH, and some of the genes involved in this process pass through the p38 MAPK pathway to impact the promoter region of AMH. This suggests that BMP15 may also affect the expression of AMH through the p38 MAPK pathway. Therefore, we hypothesized in this research that BMP15 can regulate AMH expression via the p38 MAPK pathway. If this hypothesis is true, AMH expression in goat GCs will change when the goat GCs are treated with an inhibitor of p38 MAPK and an siRNA specific for p38 MAPK *in vitro*.

2. Materials and methods

2.1. Animals

This study was carried out in strict accordance with the recommendations in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1978). The experimental conditions were approved by the Committee on the Ethics of Animal Experiments of Southwest University (No. [2007] 3) and the Animal Protection Law in China, and all efforts were made to minimize suffering.

For this experimental research, 40 Dazu Black goats in sexual maturity were selected from the Dazu Black Goat Farm at Southwest University, Chongqing, China. After the goats were euthanized, the ovaries were immediately excised and placed into a 0.9% saline solution with penicillin (100 IU/mL) and streptomycin (50 mg/mL) at 37 °C.

2.2. Isolation and culture of granulosa cells

The ovaries were washed three times with PBS after sterilization with 75% alcohol for 30 s. The cumulus-oocyte complexes (COCs) were discarded after the granulosa cells that were collected from 1- to 5-mm follicles were seeded at 10^6 viable cells/well in 6-well plates and then cultured in DMEM/F12 medium (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS) (ExCell Bio, Australia), 100 IU/mL penicillin, and 50 mg/mL streptomycin for 24 h at 37 °C with 5% CO_2 before treatment, according to a previously described method [29]. Next, after washing the cells with PBS, the culture medium was replaced with serum-free medium supplemented with or without recombinant human (rh) BMP15 (R&D Systems) (10 ng/mL or 100 ng/mL) for 48 h. The cells were collected and processed for western blot analysis or real-time PCR assays.

2.3. p38 MAPK inhibitor treatment of granulosa cells

To evaluate the effects of the p38 MAPK pathway on AMH expression, GCs that had been cultured for 24 h were washed twice with PBS and were then treated using fresh serum-free medium with or without 20 μM SB203580 (an inhibitor of p38 MAPK phosphorylation) (Beyotime, Jiangsu, China) for 2 h before the addition of 10 ng/mL BMP15. To confirm the optimum concentration of the p38 MAPK inhibitor, 6 concentrations (0 μM, 5 μM, 10 μM, 15 μM, 20 μM, 25 μM) were selected to treat the GCs. After culturing for 48 h with BMP15, GCs were collected for characterization using western blot analyses or real-time PCR assays. Conditioned culture media were collected and stored at −80 °C until the assessment of AMH levels using an ELISA (Mlbio, Shanghai, China).

2.4. Knockdown of p38 MAPK expression by siRNA in cultured granulosa cells

Three small interfering RNAs (siRNAs) specific for *p38 MAPK* and an RNAi negative control were designed and synthesized by RIBOBIO (Guangzhou, China). The siRNA sequences for p38 MAPK are shown in Table 1. The siRNAs were transfected into granulosa cells according to the manufacturer's instructions. The transfection medium was replaced 48 h later with fresh serum-free medium with or without 10 ng/mL BMP15, and the cells were further cultured for 48 h. At the end of the culture period, the conditioned culture media were collected and stored at −80 °C until AMH levels were measured using an ELISA (Mlbio). Cells were collected and processed for western blot analyses or real-time PCR assays.

2.5. RNA isolation, reverse transcription and real-time PCR

Total RNA was extracted from GCs using the RNAprep Pure Cell/Bacteria Kit (Tiangen, Beijing, China). The RNA concentration was 1000 ng/μL as measured using a Nano-100 Micro-spectrophotometer (Gene-Science Scientific Instruments Inc., USA). Reverse transcription was performed using the iScript™ cDNA Synthesis kit (Bio-Rad, CA, USA) and 2 μg of RNA. qPCR was performed using the SsoAdvanced™ SYBR Green Supermix (Bio-Rad) and the CFX96™ Real-Time System (Bio-Rad) according to the manufacturer's protocols. The AMH, SOX9 (SRY-related HMG box 9), and p38 MAPK (p38 mitogen-activated protein kinase) mRNAs were quantified. qPCR was performed in a 10-μL reaction system containing 1 μg of total RNA, and β-actin was used as an internal reference gene. The primer sequence is as shown in Table 2. The difference in the gene expression levels between the two samples was calculated using the 2-△△Ct method.

2.6. Western blot analysis

The GCs were collected and washed twice with PBS, lysed for 30 min on ice in denaturing lysis buffer containing protease inhibitors (Beyotime) and centrifuged (12,000 × g) for 10 min at 4 °C. The protein concentration in the lysate was determined using a BCA protein assay kit (Beyotime). Twenty-five micrograms of protein was separated on a 12% SDS-PAGE (Bio-Rad) gel and was transferred to a PVDF membrane. The membrane was blocked using 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 (pH 7.6) for 1.5 h at room temperature and was then incubated overnight at 4 °C with primary antibodies against p38 MAPK (1:500, Bioss, Beijing, China), phospho-p38 MAPK (1:1000, Cell Signaling Technology, MA, USA), SOX9 (1:500, Bioworld, MN, USA), and actin (1:1000, Bioss). The blots were then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000, Beyotime) for 2 h at room temperature. The bands were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo, MA, USA) using a chemiluminescence imager (Bio-Rad).

Table 1
siRNA Sequences for *P38 MAPK*.

siRNA	Sequence (5'->3')
si-bta-MAPK14_001	Sense: GAAGCUAUCCAGACCAUUU dTdT Antisense: AAAUGGUCUGGAUAGCUUC dTdT
si-bta-MAPK14_002	Sense: GGCUCGACAUACAGAUGAU dTdT Antisense: AUCAUCUGUAUGUCGAGCC dTdT
si-bta-MAPK14_003	Sense: CCGAAGAUGAACUUUGCAA dTdT Antisense: UUGCAAAGUUCAUCUUCGG dTdT