Journal of Cell Biology and Genetics Vol. 3(1), pp. 14-21, January 2013
Available online http://www.academicjournals.org/JCBG
DOI: 10.5897/JCBG12.004
ISSN 2141-6516 ©2013 Academic Journals

Full Length Research paper

Bone morphogenetic proteins (BMP) 2, 4, 6 and 7 affect ovarian follicular development through regulation of follicle-stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR) expression in goat granulosa cells

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Accepted 29 December, 2012

Bone morphogenetic proteins (BMPs), belonging to the transforming growth factor-beta family, are crucial factors in follicular growth and development in the mammalian ovary. In this study, we examined the effects of BMP2, 4, 6 and 7 on goat granulosa cells by *in vitro* culture. The results showed that BMP2, 4 and 7 increased follicle-stimulating hormone receptor (FSHR) level in goat granulosa cells whereas they decreased luteinizing hormone receptor (LHR) level in both transcript and protein levels; however, BMP6 upregulated LHR transcript and protein level in goat granulosa cells, whereas it had no effect on FSHR level. FSHR knockdown increased granulosa cell apoptosis. Our data provided the first evidence that BMP2, 4, 6 and 7 may inhibit granulosa cell apoptosis through the regulation of FSHR and/or LHR level. These findings provided new insight into the biological functions of BMP2, 4, 6 and 7 for follicular development in goat ovary.

Key words: Granulosa cell, bone morphogenetic proteins (BMPs), follicle-stimulating hormone receptor (FSHR), luteinizing hormone receptor (LHR)

INTRODUCTION

Ovarian follicular development in mammals is controlled by gonadotrophins from the pituitary gland and autocrine/ paracrine factors produced in the ovary (Fortune, 1994; Shimizu et al., 2007). The pituitary follicle-stimulating hormone (FSH) is a key hormone in the regulation of folliculogenesis and female fertility (Baby and Bartlewski, 2011; Kumar et al., 1997, 1998). In ovary, FSH triggers cyto-differentiation and proliferation of granulosa cells (GC), ultimately resulting in the development of preovulatory follicles (Erickson, 1983; Hirshfield, 1991). Because FSH acts in an endocrine manner, the expression of FSH receptor (FSHR) in target cells is essential for modulation of follicle development by FSH.

It is well known that FSHR is not expressed until midway through follicle development. In mature follicles, maintenance of FSHR expression is required to avoid death by atresia (Kobayashi et al., 1990; Yamoto et al., 1992). Therefore, it is important to elucidate the mechanism responsible for the regulation of FSHR expression to better understand the process of folliculogenesis.

Among the autocrine/paracrine factors, members of transforming growth factor beta (TGF-β) super family act most prominently in the regulatory events of follicular development. Bone morphogenetic proteins (BMPs) comprise the largest subgroup of ligands in the TGF-β super family and are associated with follicular

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development (Shimasaki et al., 2004; Shimizu et al., 2007). In ovary, BMP2 is expressed in GCs, BMP6 is expressed in oocytes and GCs , BMP4 and BMP7 are secreted by theca cells of the antral follicle (Erickson and Shimasaki, 2003; Glister et al., 2005; Knight and Glister, 2006; Shimizu et al., 2006), and their receptors are expressed in GCs (Jayawardana et al., 2006; Shimizu et al., 2006), which may indicate important potential functions for the follicular development.

Extensive studies have been done about BMPs effects on folliculogenesis and regulation of FSHR and LHR. BMP2 inhibited progesterone production of GCs in sheep (Juengel et al., 2006), rat (Inagaki et al., 2009) and human GC-like tumor cell line, KGN cell (Miyoshi et al., 2006). BMP2 up-regulated FSHR and aromatase messanger ribonucleic acid (mRNA), whereas it down-regulated LHR and StAR mRNA in human GCs (Shi et al., 2011). The theca-derived factors BMP4 and BMP7 enhanced estradiol and inhibited the progesterone production induced by FSH in rat primary GCs (Lee et al., 2001; Shimasaki et al., 1999), but in bovine GCs, BMP4 and BMP7 enhanced basal and IGF-stimulated (not FSH-induced) estradiol, while progesterone production was inhibited (Glister et al., 2004; Knight and Glister, 2006). BMP6 inhibited FSH-and/or IGF-induced progesterone production in rat, bovine and ovine GCs (Glister et al., 2004; Juengel et al., 2006; Miyoshi et al., 2007; Otsuka et al., 2001), at the same time, BMP6 decreased FSHR mRNA level in rat GCs (Juengel et al., 2006); whereas it enhanced FSHR mRNA level in human GCs (Shi et al., 2009). Furthermore, BMP6 prevented apoptosis of bovine cumulus cells, which are the GCs associated with oocytes (Hussein et al., 2005).

From these reported results, it seems that BMP2, 4, 6 and 7 have different functions in different species for the production of GC, but no information is available about the effect of BMP2, 4, 6 and 7 on goat primary GC. In this study, we investigated the effect of BMP2, 4, 6 and 7 on goat GC development, aiming at elucidating possible roles of BMP2, 4, 6 and 7 for the follicular development in goat ovary.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's/F12 medium (DMEM/F12), amphotericin B, streptomycin, phosphate-buffered saline (PBS), and Hoechst 33342 were purchased from Sigma Chemical Co., St. Louis, MO, USA. Fetal calf serum (FCS) was obtained from Biowest, Rue de la Caille, Muaille, France. Recombinant bovine BMP2, 4, 6 and 7 were purchased from R & D systems.

Granulosa cell culture

Ovaries were obtained from goats at a local abattoir. GCs from small follicles (under 5 mm in diameter) were collected by aspiration, and filtrated through a stainless steel filter (45 µm, Tokyo Screen Co., Ltd., Tokyo, Japan) to remove oocytes. Then, cells were centrifuged at 35 g and washed twice in culture medium (DMEM/F12 medium containing amphotericin B (10 µl/ml), gentamicin (5 µl/ml), and bovine serum albumin (1 mg/ml)). GCs were suspended in culture medium containing 10% FCS, and seeded in 24 well culture plates (Nalgene Nunc) at 10^5 cells/well, and cultured for 24 h at 37°C in 5% CO_2 and then the wells were washed twice with PBS to remove unattached cells. The culture medium was replaced with serum-free medium supplemented with BMP2, 4, 6 and 7 (0, 10, 50, or 100 ng/ml), respectively, and the cultures were continued for 48 h.

Hoechst 33342 staining

Hoechst staining was performed to comfirm the apoptotic profile caused by morphological changes in the nucleus, in which Hoechst 33342 binds specifically to AT base regions in DNA and emits fluorescence. After 48 h of culture, the cells from each well were rinsed in Phosphate buffered saline (PBS) and fixed with 1% glutaraldehyde (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) in PBS at 4°C for 24 h. Then, the cells were stained with Hoechst 33342 (50 µg/µl) in PBS for 3 min. The proportion of cells with nuclear fragmentation was calculated by counting the number of stained cells.

RNA extraction and qRT-PCR

Total RNA was extracted from cultured cells with TRIZOL® reagent according to the instructions provided and was frozen at -80°C in RNA storage solution. The SuperScript First-Strand Synthesis SuperMix for reverse transcription polymerase chain reaction (qRT-PCR) (Invitrogen, CA, USA) was used for cDNA synthesis. The reverse transcription conditions consist of 10 min annealing at 25°C, 60 min cDNA synthesis at 42°C, and 15 min inactivation at 70°C. For qRT-PCR, primers were designed based on *Capra hircus* FSHR (GenBank No. EU847288.1) and LHR (GenBank No. FJ755812.1) gene sequences. qRT-PCR was carried out with the SYBR Premix *Ex Taq* (Perfect Real Time) (TaKaRa, Japan) in a final volume of 20 µl including SYBR Premix *Ex Taq* (2 ×) 10 µl, 0.5 µl of each primer, and 1 µl of 2-fold dilution of first strand cDNA. qRT-PCR was performed using an iQ5 Real-Time PCR system (Bio-Rad, CA, USA). Cycling conditions were 95°C (5 min), followed by 45 cycles of 95°C (15 s), 60°C (20 s) and 72°C (30 s), finally, a dissociation curve to test PCR specificity was followed. The results were analyzed by LinRegPCR software which takes into account amplification efficiencies, and the gene (*GAPDH*) was used as a reference for normalization. Primers (Table 1) were designed using the online qPCR primer design tool QuantPrime (http://www.quantprime.de/)(Arvidsson et al., 2008

FSHR knockdown and transduction of GC

FSHR silencing was achieved by transducing GCs with Lentilox 3.7 derived plasmid containing siRNA (Tiscornia et al., 2006a). Five siRNAs were selected from the web-based prediction result (www.ambion.com) to test the silence efficiency. One of the siRNA (5'-GCCGATCTCTGCATTGGAATC-3') functions effectively to silence FSHR. Oligonucleotides containing the siRNA sequence, 9 nucleotide spacers (5'-TTCAAGAGA-3') and a polyT terminator sequence were annealed and then cloned into the HapI and XhoI sites of pLentilox 3.7 vector. siRNA was under control of the U6 promoter. High titer lentivirus production was prepared as described (Tiscornia et al., 2006b). For transduction of GCs, GCs (2×10^6) were mixed with the concentrated lentiviral supernatant, with a

Table 1. Primers used for qRT-PCR

Gene	Sequence (5´→3´)	Product size (bp)
FSHR	F: GGATTTGGAGACCTGGAGAA R: CATGTAGTTTGGGCAGGTTG	94
LHR	F: AATATCGAGCTGAACT TT R: TT GCCT GATGTGCCTAAC	286
GAPDH	F: TCATTGACCTTCACTACATGGTC R: ATGGCCTTTCCATTGATGACG	112

multiplicity of infection of 10 to 15 in a total volume of 400 µl GC medium containing 10 µg/ml polybrene (Sigma), and then centrifuged at 1000 × g for 1 h at room temperature. After 12 h incubation, 400 µl GC medium was added. GCs were transduced again with the same concentrated viral supernatant, 48 h later, to get high transduction efficiency.

Protein extraction and western blotting

For protein extraction, GC was harvested and lysed in 500 µl extraction buffer [50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.6), 150 mM NaCl, 10% glycerol (w/v), 0.5% sodium deoxycholate (w/v), 1 mM Dithiothreitol (DTT), 1 mM phenylmethanesulfonylfluoride (PMSF), complete mini protease inhibitor cocktail (Roche Applied Science)]. After centrifugation at 16000 g for 20 min at 4°C, supernatant was collected for western blotting. The protein concentration was quantified using Bio-Rad protein assay kit (BioRad, CA, USA). For immunoblotting, 50 µg protein was fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10% resolving gel) and electro-transferred to a polyvinylidene difluride (PVDF) membrane. The membrane was blocked with 5% nonfat milk for 1 h and incubated with the rabbit anti-FSHR and anti-LHR polyclonal antibodies (Invitrogen, CA, USA) diluted in 1:1000 and 1:500 ratios, respectively, overnight, then probed with a mouse anti-rabbit IgG-horseradish peroxidase conjugated antibody (1:5000) (Invitrogen, CA, USA) for 2 h at room temperature. Blots were developed using the Pierce enhanced chemiluminescence (ECL) western blotting substrate system (Thermo Fisher Scientific, Beijing, China) and the signal intensity was quantified by Quantity One analysis software (BioRad, CA, USA). β-Actin was used as a reference protein for normalization.

RESULTS

The effects of BMP2, 4, 6 and 7 on apoptosis of GCs

The effects of BMP2, 4, 6 and 7 on the incidence of GC apoptosis were determined by Hoechst 33342 staining (Figure 1). The percentage of apoptotic cells decreased significantly with treatment of BMP2, BMP4, BMP6 and BMP7 at 100, 50, 50 and 1 ng/ml, respectively, compared to the controls (Figure 1A and B). Even though all of them could inhibit GC apotosis, the optimal inhibitory concentrations are different.

The effects of BMP2, 4, 6 and 7 on the level of FSHR and LHR

In goat GC, BMP2 (100 ng/ml) significantly increased FSHR mRNA expression (Figure 2A), while it significantly decreased LHR mRNA expression (Figure 3A). BMP4 (50 ng/ml) significantly increased FSHR mRNA expression (Figure 2B), while it significantly decreased LHR mRNA expression (Figure 3B). BMP7 (1 ng/ml) significantly increased FSHR mRNA expression (Figure 2D), while it significantly decreased LHR mRNA expression (Figure 3D). Only BMP6 (50 ng/ml) significantly increased LHR mRNA expression (Figure 3C), while it had no effect on FSHR mRNA expression (Figure 2C). To confirm that the protein level of FSHR and LHR was also changed by BMPs treatment, we detected the protein of FSHR and LHR by western blot (Figure 4). Consistent with the qPCR result, the protein of FSHR was increased with the treatment of BMP2 (100 ng/ml), BMP4 (50 ng/ml) and BMP7 (1 ng/ml), but BMP6 (50 ng/ml) had little effect. LHR protein was decreased by the treatment of BMP2 (100 ng/ml), BMP4 (50 ng/ml) and BMP7 (1 ng/ml), while BMP6 (50 ng/ml) had only very weak effect. Together, these results indicate that BMP2, 4, 6 and 7 have different effects on FSHR and LHR at both transcriptional and protein level.

FSHR knockdown increases GC apoptosis

To make sure BMPs exert effect on GC apoptosis through the regulation of FSHR, we checked the GC apoptosis state in the FSHR knockdown lines. As shown in Figure 5, the transcript and protein of FSHR were both reduced in the knockdown cells (Figure 5B and D), while the number of apoptotic cells increased in the knockdown cells (Figure 5A and C), therefore, we conclude that FSHR has a positive effect against GC apoptosis.

DISCUSSION

Bone morphogenetic proteins (BMPs), belonging to the

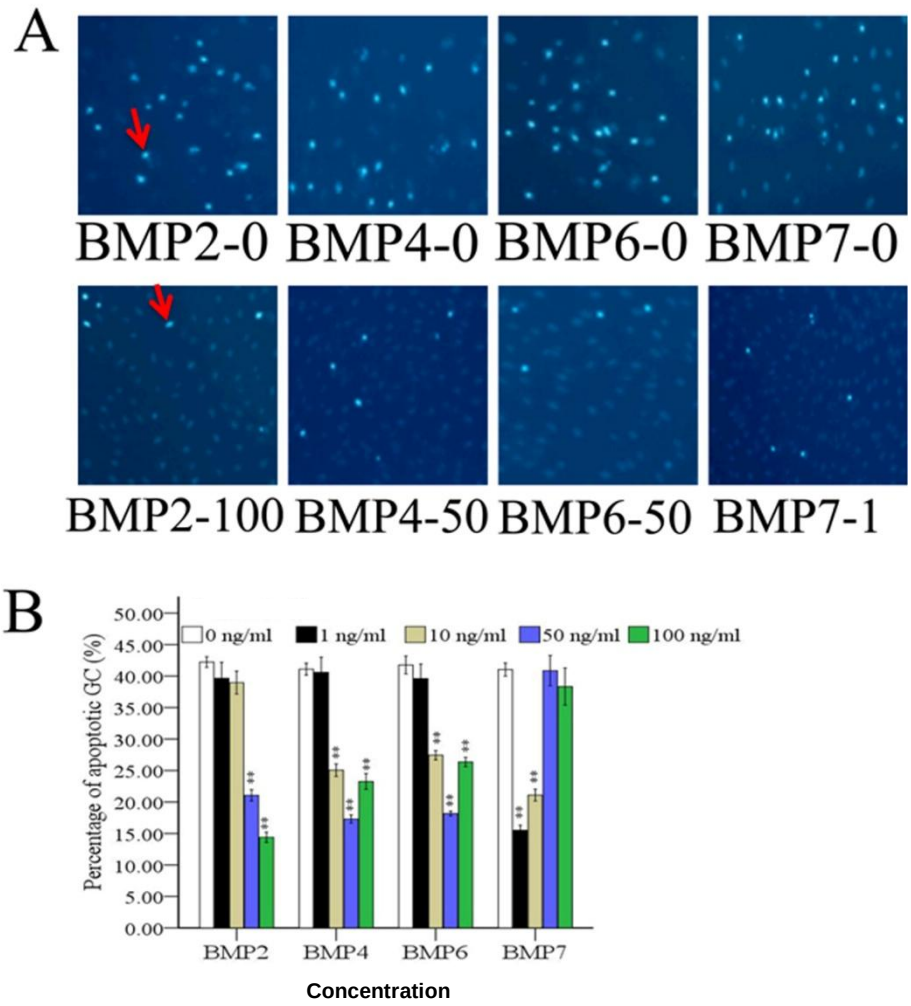

Figure 1. Effect of BMP2, 4, 6 and 7 on goat GC apoptosis. The GCs were cultured with BMP2, 4, 6 and 7 at various concentrations for 48h, then, stained with Hoechst 33342 for 3 min. A, GCs were treated with BMP2, 4, 6 and 7 at concentration of 100, 50, 50 and 1 ng/ml, respectively (bottom row). Upper row are controls without treatment. Bright spots (red arrow) are apoptotic cells. B, quantification results of apoptotic cells. Each value represents the mean ± SEM of three biological triplicates. **P < 0.01 and *P < 0.05.

transforming growth factor-beta family, are crucial factors in follicular growth and development in the mammalian ovary. The BMP subfamily members, BMP2, 4, 6 and 7 have been shown to be expressed by follicular cells in several species such as mice, rats, chickens, cows and sheep (Erickson and Shimasaki, 2003; Glister et al., 2004; Juengel et al., 2006; Onagbesan et al., 2003; Shimasaki et al., 1999). In this study, we examined the effects of BMP2, 4, 6 and 7 on goat GC apoptosis. The results show that BMP2, 4, 6 and 7 significantly inhibited GC apoptosis with different concentrations.

It was reported that BMP6 increased goat follicular and oocyte diameter but negatively affected the survival and ultrastructure of goat primordial follicles (Araújo et al., 2010). BMP7 reduced the number of primordial follicles, but the number of primary, preantral and antral follicles increased in rat ovary (Shimasaki et al., 1999). Similarly, BMP4 has been shown to increase the number of developing preantral follicles in cultured neonatal rat ovaries (Nilsson and Skinner, 2003). A drastic decrease in the expression of theca-derived BMP4 and BMP7 was the striking feature of atresia in rat ovaries (Shimasaki et al., 2004), and BMP7 reduced the incidence of cumulus cell apoptosis (Hussein et al., 2005), BMP4 and BMP7 also inhibited the bovine GC apoptosis (Kayamori et al., 2009).

The BMP family members are important for folliculogenesis in many species, and there is a growing recognition that BMPs contribute to folliculogenesis by inhibiting luteinization of GCs (Shimasaki et al., 2004). In the present study, we found that BMP2, 4 and 7 induced FSHR mRNA expression in goat GCs at different

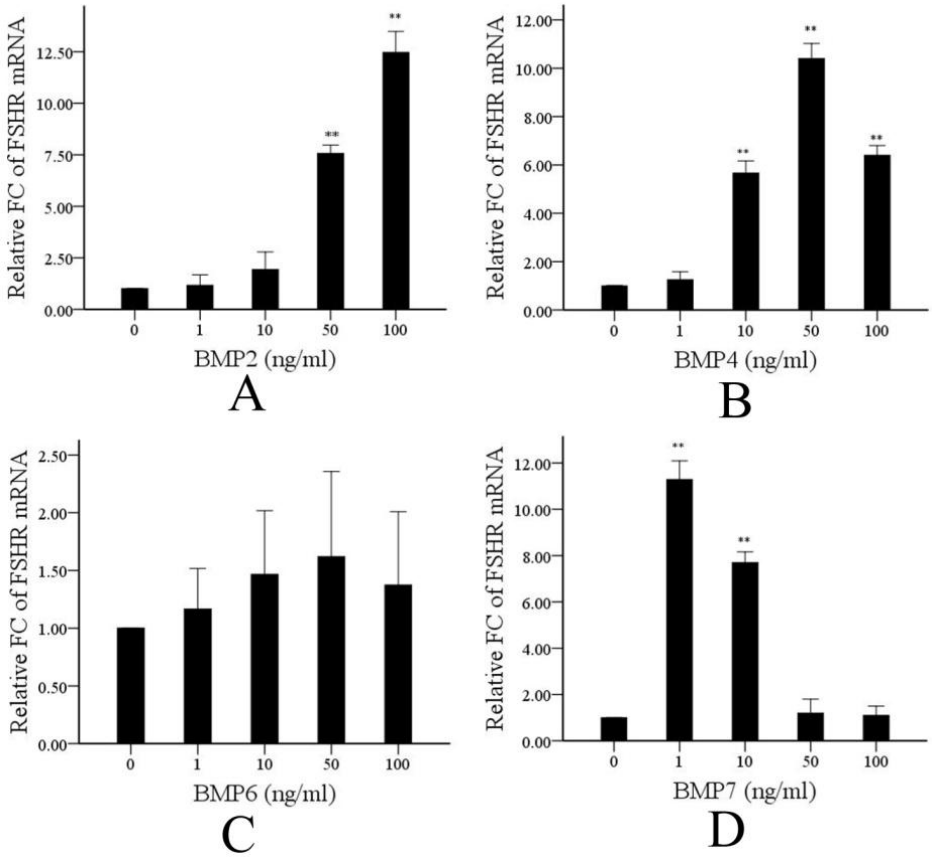

Figure 2. Effect of BMP2, 4, 6 and 7 on FSHR mRNA expression level. The goat GCs were cultured with BMP2, 4, 6 and 7 at various concentrations for 48 h. Total RNA was extracted from the GC and subjected to qRT-PCR. The results are relative fold changes (FC) to controls, all data are from three biological replicates. GAPDH mRNA level was used as an internal control for normalization. Data are presented as mean ± SEM. **P < 0.01 and *P < 0.05.

concentrations, which indicates that BMP2, 4 and 7 may contribute to increasing FSH sensitivity of GCs and then promote folliculogenesis. Our finding is consistent with that in human GC, because it was reported that BMP2 (100 ng/ml) significantly increased FSHR and aromatase mRNA expression, while BMP2 significantly decreased LHR mRNA expression (Shi et al., 2011), similarly, BMP7 induced FSHR mRNA expression, but decreased LHR mRNA expression (Shi et al., 2010).

Lee et al. (2004) also reported that BMP7 increased FSHR mRNA in mouse neonatal ovary, but BMP7 treatment inhibited expression of mRNA of LHR, which is a key factor required by GCs to undergo luteinization (Pangas et al., 2006). The function of BMP6 in folliculogenesis appears to differ in human and ruminants versus rodents (Glister et al., 2004; Otsuka et al., 2001). BMP6 decreased FSHR, inhibin-A and inhibin/activin-B subunits mRNA expressions in rat GC (Otsuka et al., 2001), whereas BMP6 enhanced inhibin-A and activin-A production in bovine GC (Glister et al., 2004). In cultured human GC, BMP6 stimulated gene expression of the inhibin/activin-B a and b subunits, but not the inhibin-A subunit. BMP6 also stimulated the expression of FSHR and Anti-Müllerian hormone (AMH) in cultured human GC. In addition, BMP6 gene expression in cultured human GC was increased by activin-A and BMP7 (Shi et al., 2009). In our study, BMP6 (50 ng/ml) significantly increased LHR mRNA expression, while it had no effect on FSHR mRNA expression.

On the basis of our data, we propose that BMPs (BMP2, 4, 6 and 7) may be associated with follicular selection during follicular development in goat ovary. Implying that there is a large amount of BMP2, 4, 6 and 7 in theca cells at the time of deviation, and then follicle is predestined to grow to the ovulatory follicle. If follicle did not express BMP2, 4, 6 and 7 at the time of deviation, the follicles would undergo atresia.

Conclusion

This study provides the first evidence that BMP2, 4, and

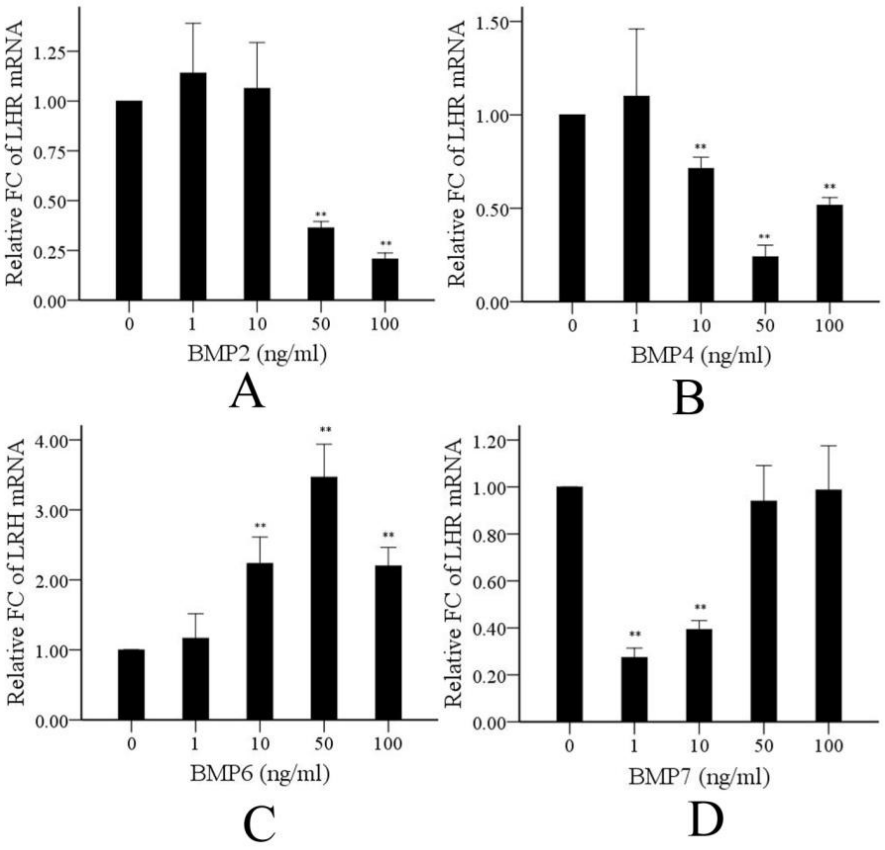

Figure 3. Effect of BMP2, 4, 6 and 7 on LHR mRNA expression level. The goat GCs were cultured with BMP2, 4, 6 and 7 at various concentrations for 48 h. Total RNA was extracted from the GC and subjected to qRT-PCR. The results are relative fold changes (FC) to controls, all data are from three biological replicates. GAPDH mRNA level was used as an internal control for normalization. Data are presented as mean ± SEM. **P < 0.01 and *P < 0.05.

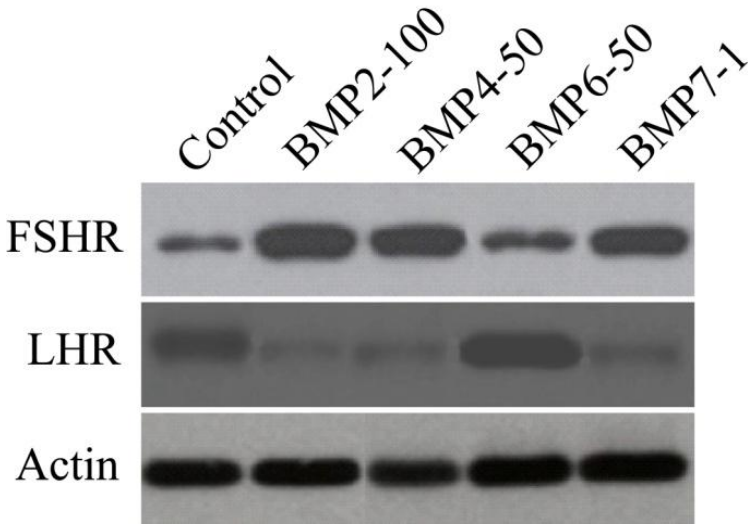

Figure 4. Western blot result of FSHR and LHR with the treatment of BMP2, BMP4, BMP6 and BMP7. The goat GCs were cultured with BMP2 (100 ng/ml), BMP4 (50 ng/ml), BMP6 (50 ng/ml) and BMP7 (1 ng/ml) for 48 h, protein were extracted for protein blot with FSHR and LHR antibodies. Protein from untreated cells was used as control. Actin was used as a loading control.

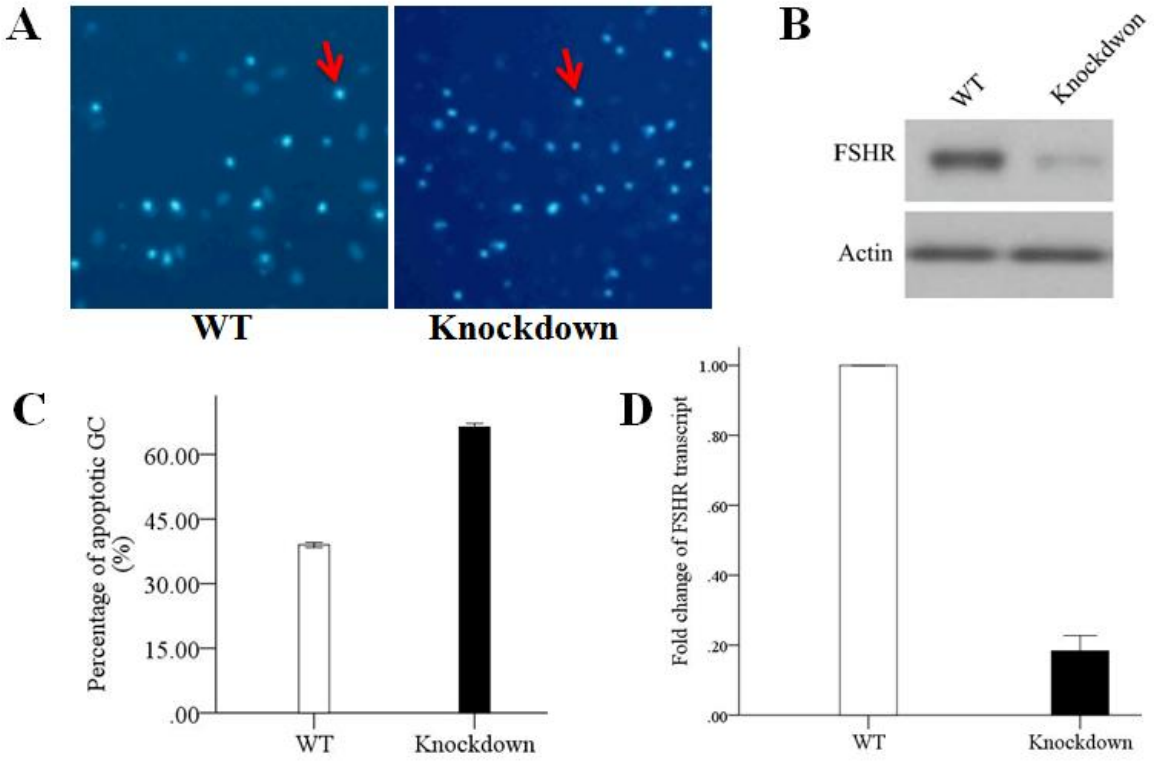

Figure 5. Effect of FSHR knockdown on GC apoptosis. A, hoechst staining of GC from WT and a knockdown line. The apoptotic cells were indicated with red arrow. B, western blot result of FSHR in WT and knockdown cells to confirm FSHR protein was reduced. C, quantification result of A. D, qPCR result of FSHR transcript from WT and knockdown cells.

7 inhibit goat GC apoptosis through upregulation of FSHR mRNA and causes downregulation of LHR mRNA in goat GCs. Only BMP6 significantly increased LHR mRNA expression, while it had no effect on FSHR mRNA expression in goat GCs. Cultured GCs used in this study may not represent the stages of growing follicles and further studies are needed. However, our finding that BMP2, 4, 6 and 7 regulate folliculogenesis-related genes in goat GC is novel, and is an open new insight into our understanding of ovary physiology.

ACKNOWLEDGEMENTS

This work was supported by National High Technology Research and Development Program (863 Program) of China (No. 2007AA10Z167) and Jiangshu province Three New Projects of Agriculture (No. SXGC[2012]051).

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