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Tissue-specific mRNA expression profiles of *GDF9*, *BMP15*, and *BMPR1B* genes in prolific and non-prolific goat breeds

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ABSTRACT: The tissue-specific mRNA expression profiles of *GDF9*, *BMP15*, and *BMPR1B* genes in goats were investigated. The mRNA expression of the genes was detected in prolific Jining Grey goats and non-prolific Liaoning Cashmere goats by reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR. The results showed that *GDF9* and *BMPR1B* were widely expressed in 20 tissues, while *BMP15* gene was expressed exclusively in ovary and pituitary. The expression levels of *GDF9* and *BMPR1B* genes were the highest in ovary. The expression level of *BMP15* gene in the ovary of Jining Grey goat was 4.96-fold higher than that in Liaoning Cashmere goat ($P < 0.05$), but there were no differences ($P > 0.05$) in the expression level of *GDF9* and *BMPR1B* genes. This is the first study to analyze the tissue expression pattern of *GDF9*, *BMP15*, and *BMPR1B* genes in goat, and the *BMP15* might be a major gene for the prolificacy of Jining Grey goat.

Keywords: goat; prolificacy; ovary; RT-PCR; gene expression

INTRODUCTION

Prolificacy is one of the most important traits in goat and is regulated by some specific genes. Bone morphogenetic protein 15 (*BMP15*), growth differentiation factor 9 (*GDF9*), and bone morphogenetic protein receptor, type IB (*BMPR1B*), known as prolificacy candidate genes, play key roles in regulating ovarian functions in animals (Juengel et al. 2002; Knight and Glister 2003). In sheep, spontaneous mutations of *BMP15* ($FecX^I$, $FecX^H$, $FecX^G$, $FecX^B$, $FecX^L$, and $FecX^R$) showed that ovulation rate in the heterozygotes was increased, but homozygous mutants were completely sterile (Galloway et al. 2000; Hanrahan et al. 2004; Bodin et al. 2007; Martinez-Royo et al. 2008; Monteagudo et al. 2009). The mutations of *GDF9* ($FecG^H$, $FecG^T$, $FecG^E$, $FecG^F$, and $FecG^V$) had introduced non-conservative amino acid changes reported to be associated with the litter size of sheep (Melo et al. 2008; Nicol et al. 2009; Silva et al. 2011; Vage et al. 2013; Souza et al. 2014). A variation (*FecB*) in *BMPR1B* caused substitution of one amino acid (arginine to glutamine) and increased the ovulation rate of ewes (Mulsant et al. 2001; Souza et al. 2001; Fabre et al. 2003). It is obvious that *GDF9*, *BMP15*, and *BMPR1B* genes are all closely related with ovine reproduction. Hence, investigating the mRNA expression levels of these genes in goats should be very helpful.

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Until now, the mRNA of *GDF9* and *BMPR1B* has been detected in different ovine tissues (Yang et al. 2009; Hu et al. 2010). However, in the study of goats, most attention has been focused on ovaries (Cui et al. 2009; Yang et al. 2012; Pramod et al. 2013), and information on the tissue expression pattern of *GDF9*, *BMP15*, and *BMPR1B* genes is scant. Jining Grey goat is an excellent local breed for its significant characteristics of hyper-prolificacy, while Liaoning Cashmere goat is a non-prolific breed renowned for its fine wool. The averaged litter size of Jining Grey goat is 2.94 while in Liaoning Cashmere goat it is 1.18 (Tu 1989). To explore the potential role of *GDF9*, *BMP15*, and *BMPR1B* in goats, we compared the tissue expression profile and the mRNA expression levels of these genes between Jining Grey goat and Liaoning Cashmere goat in different tissues using reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR.

MATERIAL AND METHODS

Experimental materials. Six Jining Grey goats (twins in three consecutive parities) and six Liaoning Cashmere goats (single-birth animals in three consecutive parities) were supplied by the Jining Grey Goat Conservation Base (Jiaxiang county, Shandong Province, China) and the Liaoning Cashmere Goat Breeding Center (Liaoyang City, Liaoning Province, China) during the breeding season (April to May), respectively. All goats, healthy, aged 3–4 years, were kept in a sheltered outdoor paddock and were provided with alfalfa hay and water available *ad libitum*. The estrous cycles of all adult goats were synchronized with intravaginal sponges impregnated with fluorogestone acetate (FGA; 40 mg) (Intervet, Angers, France) inserted for 14 days. Estrus-synchronized goats were slaughtered during the follicular phase 36 h after FGA sponge removal. Samples of twenty tissues were taken from different parts of each animal's body (heart, liver, lung, skeletal muscle, kidney, spleen, subcutaneous fat, adrenal, ovary, hypothalamus, pituitary, uterus, uterine horn, oviduct, cerebral cortex, cerebellum, thalamus, hippocampus, pons, and medulla oblongata). All samples were snap-frozen in liquid nitrogen and then stored at −80°C until RNA extraction.

All experimental procedures involving animals were approved by the Chinese Ministry of Agriculture and the animal care and use committee at the institution where the experiments were performed.

Primers design. Using Primer Express (Version 2.0, 1995) software, the *GDF9*, *BMP15*, *BMPR1B*, and *GAPDH* RT-PCR primers and the real-time PCR primers were designed based on the sequences of JN100108, JF824148, KF758765, and AJ431207.1 (http://www.ncbi.nlm.nih.gov/) in GenBank and

Table 1. Primer sequences of reverse transcription (RT) PCR and real-time PCR

	Gene name	Primer sequence (5'→3')	Product size (bp)	Annealing temperature (°C)
RT-PCR primer	*GDF9*	F: AGGCATACGCTACCAAGGAGG R: CCACAACAGTAACACGATCCAGG	171	58
	BMP15	F: TGACGCAAGTGGACACCCTA R: GAAGAAGGAAAGTGATTGGTTGGG	266	56
	BMPR1B	F: AAGAAGCCAGACCTCG R: CCACTTTCCCATCCAA	212	55
	GAPDH	F: CATGGTCTACATGTTCCAGTATGATTC R: TCACCATCTTCCAGGAGCG	114	56
Real-time PCR primer	*GDF9*	F: TAAAGGTTCTGTATGATGGG R: AGCCGAACAGTGTTGTAGAG	151	60
	BMP15	F: CGCTGGCTAGTGTAGCAAGG R: GTGGGAATGAGTTAGGTGAAG	143	60
	BMPR1B	F: CCTGTGGTCACTTCTGGATGTC R: TTCCGTTCTGTGCAGCATTC	107	60
	GAPDH	F: GAGAAACCTGCCAAGTATGA R: CGAAGGTAGAAGAGTGAGTG	139	60

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synthesized by Shanghai Invitrogen Biotechnology Co., Ltd. *GAPDH* was used as an internal control to normalize all of the threshold cycle (Ct) values. All primer sequences are listed in Table 1.

RNA extraction and cDNA synthesis. Total RNA was extracted from caprine tissues (each tissue smashed, mixed, and 50–100 mg chosen for RNA extraction) using Trizol reagent according to the instructions of the manufacturer (TaKaRa, Dalian, China). The RNA samples were treated with DNase using TURBO DNA-free™ Kit (Ambion, Austin, USA). RNA quality and quantity were assessed by agarose gel electrophoresis and UV-1201 spectrophotometer (Shimadzu, Kyoto, Japan), respectively. 500 ng of total RNA was used for reverse transcription (PrimeScript™ RT reagent kit; TaKaRa) using random hexamers as primers and following manufacturer's recommendations. Synthesized cDNA was stored at –20°C until further use.

Reaction system and conditions for RT-PCR and fluorescence quantitative PCR. The cDNA of all tissue samples (heart, liver, lung, skeletal muscle, kidney, spleen, subcutaneous fat, adrenal, ovary, hypothalamus, pituitary, uterus, uterine horn, oviduct, cerebral cortex, cerebellum, thalamus, hippocampus, pons, and medulla oblongata) was used for RT-PCR analysis. The RT-PCR reactions were carried out in a 20 μl volume including 2× PCR Master Mix (Biomed, Beijing, China) 10 μl, each primer (10μM) 0.3 μl, cDNA 1.5 μl, ddH_2O 7.9 μl. Amplification conditions were as follows: initial denaturation at 95°C for 5 min; followed by 32 cycles of denaturation at 95°C for 30 s, annealing for 30 s at appropriate temperatures (Table 1), extension at 72°C for 30 s; with a final extension at 72°C for 5 min on Mastercycler 5333 (Eppendorf AG, Hamburg, Germany). The PCR products were cloned and sequenced as described previously (Huang et al. 2013).

Considering that *GDF9*, *BMP15*, and *BMPR1B* are all reproduction related genes, four reproductive tissues (hypothalamus, pituitary, ovary, and uterus) were selected for further qPCR analysis. Real-time PCR amplification was performed in 20 μl of reaction mixture containing 2 μl of cDNA, 0.4 μl of each forward and reverse primer (10μM), 0.4 μl of ROX Reference Dye II (50×), 10 μl of SYBR® Green Real-time PCR Master Mix (2×), and 6.8 μl of ddH_2O. The reaction carried out without template was used as blank. PCR amplification was performed in triplicate wells, using the following conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and of 60°C for 34 s. The melting curve was analyzed after amplification. A peak of the anneling temperature at 85°C ± 0.8 on the dissociation curve was used to determine the specificity of PCR amplification.

Data processing and analysis. The $2^{-\Delta\Delta Ct}$ method was used to process the real-time PCR results (Livak and Schmittgen 2001). Statistical analyses were carried out using SPSS software (Version 15.0, 2009), and two-tailed paired Student's *t*-test was performed between the two breeds.

RESULTS

Purity and integrity of total RNA. Total RNA samples were assayed using 1% agarose gel electrophoresis. Three bands (representing 28S, 18S, and 5S) were observed with no DNA contamination or significant degradation. The A_{260}/A_{280} ratios of the samples were 1.8–1.9. Therefore, the total RNA isolated was shown to be of sufficient purity for the purposes of reverse transcription.

Tissue expression profiling of GDF9, BMP15, and BMPR1B genes. The result of agarose gel electrophoresis showed that the products from each of the RT-PCR amplifications exhibited a single band of the expected size (Figure 1). In addition, sequencing results verified that the sequences were correctly amplified.

As shown in Figure 1 (listing only one figure of each breed), both in Jining Grey goat and Liaoning Cashmere goat, *GDF9* and *BMPR1B* genes were widely expressed, but *BMP15* gene was expressed exclusively in ovary. Compared to *GAPDH* expression, the caprine *BMPR1B* was moderately expressed in 7 tissues (ovary, hypothalamus, pituitary, uterus, uterine horn, oviduct, and cerebral cortex), weakly in other 13 tissues. The caprine *GDF9* was weakly expressed in 4 tissues (subcutaneous fat, heart, pons, and medulla oblongata), moderately in other 16 tissues.

Expression levels of GDF9, BMP15, and BMPR1B genes. Using the established SYBR® Green real-time quantitative PCR method described above, the expression levels of *GDF9*, *BMP15*, and *BMPR1B* were determined in four tissues (hypothalamus, pituitary, ovary, and uterus) of high fecundity breed Jining Grey goat and low fecundity breed Liaoning Cashmere goat. The

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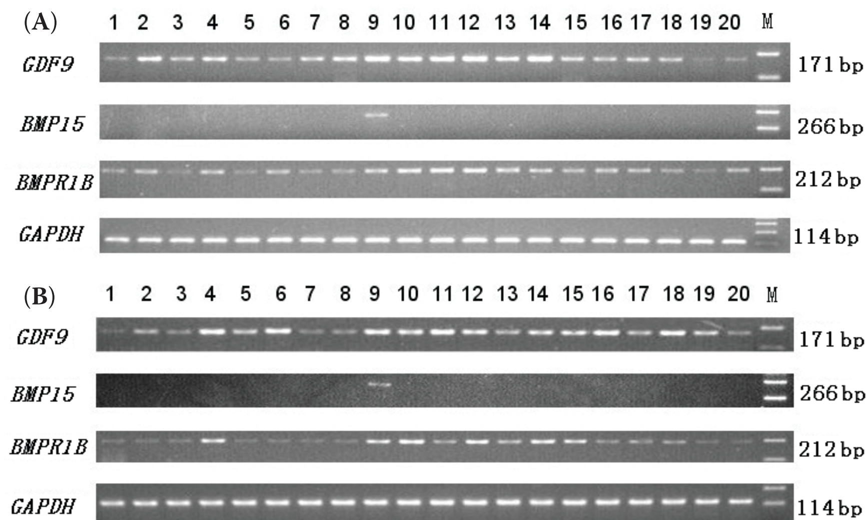

Figure 1. Reverse transcription PCR analysis of *GDF9*, *BMP15,* and *BMPR1B* expression in different tissues

Jining Grey goats (**A**), Liaoning Cashmere goats (**B**): 1 = heart, 2 = liver, 3 = lung, 4 = skeletal muscle, 5 = kidney, 6 = spleen, 7 = subcutaneous fat, 8 = adrenal, 9 = ovary, 10 = hypothalamus, 11 = pituitary, 12 = uterus, 13 = uterine horn, 14 = oviduct, 15 = cerebral cortex, 16 = cerebellum, 17 = thalamus, 18 = hippocampus, 19 = pons, 20 = medulla oblongata, M = DNA Marker I (100–600 bp) (Tiangen, Beijing)

expression level of each gene in the ovary of Liaoning Cashmere goat was defined as 1.0. As shown in Figure 2, the *GDF9* and *BMPR1B* genes were expressed in all tissues with the highest level in ovary, followed by hypothalamus, pituitary, and uterus. *BMP15* was expressed in ovary as well as pituitary where it was not detected by the RT-PCR approach. Expression of *GDF9* and *BMPR1B* exhibited no significant differences in all tissues between the two breeds ($P > 0.05$), but the expression of *BMP15* gene in ovary was significantly higher in Jining Grey goats (4.96-fold) than in Liaoning Cashmere goats ($P < 0.05$).

DISCUSSION

GDF9. The *GDF9* gene, a TGF beta superfamily member, is essential for ovarian folliculogenesis (Kaivo-Oja et al. 2003). It was firstly identified in the human ovary (McPherron and Lee 1993). Subsequent studies indicated that the *GDF9* was expressed in the ovaries of sheep (Feary et al. 2007) and goat (Silva et al. 2005). Earlier, the *GDF9* expression was detected in ten tissues (hypothalamus, pituitary, ovary, uterus, fallopian tubes, heart, liver, spleen, lung, and kidney) of Hu sheep (Hu et al. 2010). In the present study, *GDF9* was detected in all 20 tissues, which implies that it plays a role in promoting differentiations of many tissues. The highest expression of *GDF9* in ovary further confirmed that *GDF9* is associated with the ovarian function.

Results of Hu et al. (2010) showed that the *GDF9* mRNA expression level of ovary in prolific Hu sheep (triplets) was prominently higher than that in non-prolific Hu sheep (single lamb). However, in our study, there were no differences in the expression of *GDF9* between prolific and non-prolific goats ($P > 0.05$), which was consistent with previous studies (Yang et al. 2012; Pramod et al. 2013). To some extent, it was explained by the fact that the role of *GDF9* might be different between sheep and goat.

BMPR1B. The *BMPR1B* is a major gene responsible for high prolificacy firstly identified in Booroola Merino sheep (Davis et al. 1982). A previous study revealed that *BMPR1B* was expressed in reproductive tissues, brain, skeletal muscle, and kidney of sheep (Wilson et al. 2001). Recently, Yang et al. (2009) have found that *BMPR1B* was expressed in 10 different tissues (ovary, ear, spinal cord, pituitary, bone, uterus, hypothalamus, kidney, skeletal muscle, and fallopian tubes) of Chinese Merino sheep. In the present study, *BMPR1B* was broadly expressed in all caprine tissues tested. Although *BMPR1B* plays an important role in osteogenesis, *BMPR1B* had the highest expression level in ovary, which indicates that the *BMPR1B* expression is also

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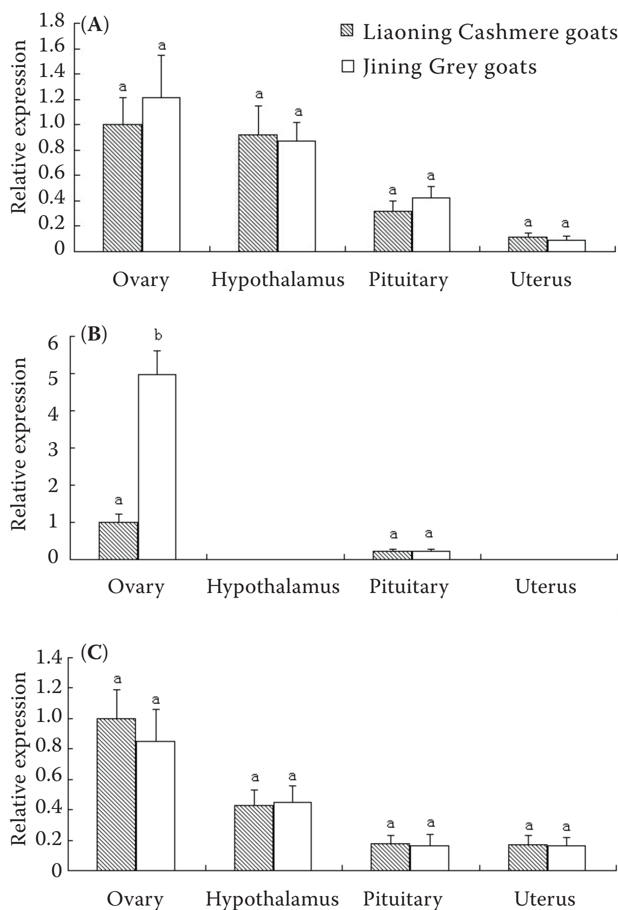

Figure 2. Expression levels of *GDF9* (**A**), *BMP15* (**B**), and *BMPR1B* (**C**) genes in ovary, hypothalamus, pituitary, and uterus

results are expressed as mean ± SEM, different letters within tissues represent significant differences ($P < 0.05$)

important for the ovarian function. Similarly to *GDF9* gene, there was no difference ($P > 0.05$) in the expression of *BMPR1B* between Jining Grey goat and Liaoning Cashmere goat, implying that *BMPR1B* may not contribute to high fecundity of Jining Grey goats.

BMP15. The *BMP15* gene plays a critical role in ovarian function (Knight and Glister 2003). Sheep lacking *BMP15* have arrested folliculogenesis at primary follicles (Juengel et al. 2002). However, there is little information about the expression level of *BMP15* in different tissues of goat. We explored the *BMP15* tissue expression in goat for the first time. In our study, *BMP15* gene was specially expressed in ovary and pituitary. Previous studies showed that *BMP15* was expressed in the pituitary of mice (Dube et al. 1998; Otsuka and Shimasaki 2002), but not in bovine (Hosoe et al. 2011). It was hypothesized that *BMP15* could play a physiological role in the monotropic rise of FSH secretion by the pituitary during the estrous and menstrual cycle (Otsuka and Shimasaki 2002). In this study, the *BMP15* mRNA was detected in pituitary (real-time PCR results), suggesting that the function of *BMP15* in pituitary might be similar between goats and mice.

Interestingly, the expression level of *BMP15* gene in the ovary of prolific Jining Grey goat was significantly ($P < 0.05$) higher than that in non-prolific Liaoning Cashmere goat. This observation was consistent with previous studies of comparison in prolific and non-prolific goat breeds (Cui et al. 2009; Yang et al. 2012; Pramod et al. 2013). It seems that prolific goats have higher expression of *BMP15* gene in ovary. *BMP15* gene might be a key gene for the prolificacy of goat. However, in the oocytes of Booroola Romney sheep, *BMP15* expression was lower in prolific than in wild animals (Crawford et al. 2011). It was because *BMP15* regulated ovulation rate and female fertility in a species-specific manner (Al-Musawi et al. 2013). *BMP15* might play a different role in sheep and goat. Our results lead to speculations that the high level of *BMP15* expression in prolific goats induced multiple follicles maturation while the low *BMP15* level might cause atresia in the non-prolific goats. Therefore, the prolificacy of Jining Grey goat might be due to the high expression level of *BMP15* gene.

CONCLUSION

In conclusion, both *GDF9* and *BMPR1B* were widely expressed in 20 tissues, and had no contribution to high fecundity of Jining Grey goats. However, *BMP15* gene was specially expressed in ovary and pituitary, and the ovarian expression level of *BMP15* gene in the prolific goats was higher than that in non-prolific goats ($P < 0.05$). *BMP15* might be a key gene for the prolificacy of Jining Grey goats. This was the first study to analyze the tissue expression pattern of *GDF9*, *BMP15*, and *BMPR1B* genes in goats systematically. Our study will hopefully provide a basis for the development of the functional research in these genes.

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