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Expression analysis of *BMPR1B*, *BMP15*, and *GDF9* in prolific and non-prolific sheep breeds during the follicular phase

Ji-Shun Tang[1,2], Wen-Ping Hu[1], Ran Di[1], Xiang-Yu Wang[1], Xiao-Sheng Zhang[3], Jin-Long Zhang[3], Qiu-Yue Liu[1], Ming-Xing Chu[1]**

[1]*Key Laboratory of Animal Genetics, Breeding and Reproduction of Ministry of Agriculture and Rural Affairs, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing, P.R. China*

[2]*Institute of Animal Husbandry and Veterinary Medicine, Anhui Academy of Agricultural Sciences, Hefei, P.R. China*

[3]*Tianjin Institute of Animal Sciences, Tianjin, P.R. China*

**Corresponding authors: mxchu@263.net, liuqiuyue@caas.cn*

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Abstract: To elucidate how expression characteristics of *BMPR1B*, *BMP15*, and *GDF9* are associated with sheep reproduction, tissue-specific mRNA expression of these genes in Small Tail Han (STH) ewes (a polytocous breed) and Sunite (SNT) ewes (a monotocous breed) in the follicular phase were investigated using transcription profiling and quantitative real-time PCR (qPCR). Expression levels of the three genes were all highest in ovaries of the two sheep breeds, and *BMPR1B* and *GDF9* expression in the ovarian tissue was significantly higher in STH sheep compared with SNT sheep ($P < 0.01$), whereas *BMP15* expression in pituitary, ovarian, oviduct, and uterine tissues was significantly lower in STH sheep compared with SNT sheep ($P < 0.01$). This study revealed that *BMPR1B*, *BMP15*, and *GDF9* may play important roles in the hypothalamic-pituitary-gonadal axis, especially in the ovary, which may affect sheep prolificacy. *BMPR1B* and *GDF9* expression might increase litter size, whereas *BMP15* expression may decrease litter size. These results provide preliminary information regarding molecular regulatory mechanisms underlying sheep prolificacy.

Keywords: tissue; candidate gene; transcription profiling; qPCR; prolificacy

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J.-S. Tang and W.-P. Hu contributed equally to this study.

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A majority of sheep breeds are monotocous. Effective improving the reproductive performance is an urgent issue to increase human demand for products in the sheep industry. Exploring the molecular mechanisms underlying the reproductive performance is necessary for detecting major genes that increase prolificacy. Among the candidate genes that affect sheep prolificacy, bone morphogenetic protein receptor 1B (*BMPR1B*), bone morphogenetic protein 15 (*BMP15*), and growth differentiation factor 9 (*GDF9*), as the causative genes with known target mutations, have received substantial attention (Paz et al. 2015; E1 Fiky et al. 2017). It has been reported that BMPR1B is a key transmembrane receptor protein mainly involved in the transforming growth factor β (TGFβ) pathway, while it plays a critical role in the ovarian function (Zamani et al. 2015).

BMP15 and GDF9 belong to the TGFβ superfamily; they are secreted by the oocyte and play important regulatory roles in follicular growth and differentiation through paracrine pathways (Kaivo-Oja et al. 2003). Binding of GDF9/BMP15 to their receptor complexes (e.g. TGFBR1, BMPR1B and BMPR2), which are located on granulosa cells (GC), results in phosphorylation of Smad signalling molecules, which in turn activates Smad-dependent and independent signalling pathways, and regulates the transcription of downstream target genes (Juengel et al. 2013). Therefore, there are important interactions among BMPR1B, BMP15, and GDF9 during follicular development, which further affects the lambing performance of sheep. To date, most studies of polytocous candidate genes such as *BMPR1B*, *BMP15*, and *GDF9* have mainly focused on correlations between gene mutations and litter size (Chu et al. 2007; Paz et al. 2015; Zamani et al. 2015). The mutated *BMPR1B* (*FecB*) is an autosomal dominant gene with an additive effect on ovulation rate (Jain et al. 2014; Foroughinia et al. 2017). In addition, nine mutations ($FecX^I$, $FecX^H$, $FecX^G$, $FecX^B$, $FecX^L$, $FecX^R$, $FecX^{Gr}$, $FecX^O$, and $FecX^{Bar}$) of *BMP15* and five mutations ($FecG^E$, $FecG^H$, *FecTT*, $FecG^F$, and $FecG^V$) of *GDF9* were significantly correlated with litter size in some sheep breeds (Chu et al. 2007; Souza et al. 2014; El Fiky et al. 2017). However, these mutations of *BMP15* and *GDF9* were mainly detected in polytocous sheep breeds of European countries and New Zealand, and few of them were revelaed in Chinese endemic sheep breeds, including Small Tail Han (STH) and Sunite (SNT) sheep.

In China, some local sheep breeds, such as STH and SNT sheep, are valuable for studying the molecular genetic basis of lambing performance differences. STH sheep are an important source of both wool and meat, and they also exhibit uniquely high fecundity. SNT sheep are a representative monotocous Mongolian sheep breed, well known for its good-quality meat; however, this breed has seasonal oestrus and low fecundity. Recently published research has shown that the STH sheep population can be divided into three *FecB* genotypes (*BB*, *B*+, and ++) using the TaqMan probe method (Liu et al. 2017), of which *FecB BB* and *FecB*++ are mutant and wild homozygous genotypes, respectively, while *FecB B*+ is a heterozygous genotype. The *FecB* mutation was associated with the prolificacy of STH sheep. However, some STH sheep of *FecB*++ genotype also had high fecundity and maintained stable heredity in production (Tang et al. 2019).

To our knowledge, tissue-specific expression profiles and quantitative analysis of the main organs, glands, and reproductive tissues of polytocous and monotocous sheep breeds have not been reported. Therefore, in this study, we investigated *BMPR1B*, *BMP15*, and *GDF9* expression in major organ and gonadal axis tissues of STH and SNT sheep, including oviduct and uterine tissues during the follicular phase. The preliminary results may provide a new insight into understanding the molecular regulatory mechanism for reproduction in sheep.

MATERIAL AND METHODS

Experimental sheep selection and treatment. All experimental procedures involving animals were approved by the Chinese Ministry of Agriculture and the Animal Care and Use Committee. All experiments were performed according to institutional guidelines.

The STH and SNT adult ewes without *FecB* mutation (2–3 years old) used for study were selected from Sheep & Goat Breeding Farm of Tianjin Institute of Animal Sciences (Tianjin City, China). In addition, the selected STH ewes are polytocous ewes. All sheep were kept in a sheltered outdoor paddock, and high-quality hay and clean water were provided by a designated keeper. The oestrous cycles of all ewes were synchronized with

https://doi.org/10.17221/101/2018-CJAS

progesterone (vagina embolism) using a controlled internal drug release device (CIDR, InterAg Co., Ltd., New Zealand) which was inserted into the vagina for 12 days. Then, three healthy ewes of STH breed and three healthy ones of SNT breed were chosen during the follicular phase. Tissue samples were collected 45–48 h after CIDR removal.

Tissue sample collection. Fourteen selected tissue samples included heart, liver, spleen, lung, kidney, brain, cerebellum, hypothalamus, pituitary, ovary, oviduct, uterus, adrenal gland, and duodenum; the samples were taken from both sheep breeds. The tissue samples were collected within 30 min after euthanasia. The whole ovary with a large follicle was chosen to extract tissue RNA. In STH sheep, there were 2–3 developing large follicles on the surface of the ovary with the follicles diameter 5–8 mm. In SNT sheep, there was only one large follicle and the diameter of the follicle was over 10 mm. Fresh samples were placed in 2-ml RNase-free tubes (Nunc, Denmark), immediately frozen in liquid nitrogen, and then stored at −80°C in the laboratory until use.

Primer design. Primers were designed by Primer3 (Version 4.1.0) (http://primer3.ut.ee/). Primer sequences for *BMPR1B* (Accession No. NM_001142888.2), *BMP15* (Accession No. NM_001114767.1), *GDF9* (Accession No. NM_001009431.1), and β-actin (*ACTB*) (Accession No: NM_001009784.1) for transcription profiling and quantitative real-time polymerase chain reaction (qPCR) were based on GenBank sequences (http://www.ncbi.nlm.nih.gov/). Primer sequences of *BMP15* for transcription profiling were obtained from published literature (Mandon-Pepin et al. 2003). All primers were synthesized by Tianyi Biotechnology Co., Ltd. (Beijing City, China). The reference gene *ACTB* was used as an internal control to normalize all threshold cycle (Ct) values. Primer information, including gene name, amplified fragment length and annealing temperature (T_a), is listed in Table 1.

Total RNA extraction and cDNA synthesis. Tissue RNA was extracted using the total RNA extraction kit for animal tissue (Tiangen, China), and TRIzol (Invitrogen, USA) was used as a lysis solution. During the operation, RNA preparations were treated with DNase. Quantity and quality of total RNA were inspected by 1.5% agarose gel electrophoresis and ultraviolet spectrophotometry (UV-1201; Shimadzu, Japan), respectively. Then, the qualified RNA was stored at −80°C.

The first strand of cDNA was prepared following the instructions of the PrimeScript™ RT Reagent Kit (TaKaRa, China). The reaction program was 37°C for 15 min followed by 85°C for 5 s. The reaction system contained 1 µl of PrimeScript RT Enzyme Mix I, 1 µl of Oligo dT Primer, 1 µl of random 6-mers, 4 µl of 5× PrimeScript Buffer (for Real Time), and 1 µg of total RNA, and RNase-free water was added to make the total volume 20 µl. After gentle mixing, a reverse transcription

Table 1. Transcription profiling and qPCR primers information

Methods	Gene name	Primer sequence (5'-3')	Amplicon (bp)	Annealing temperature (°C)
Transcription profiling	*BMPR1B*	F: GGGTTCTACGACTCCGCTTC R: GGTTACTTTCAGGCCCATCAT	237	60
	BMP15	F: GGGTTCTACGACTCCGCTTC R: GGTTACTTTCAGGCCCATCAT	273	62
	GDF9	F: TAGTCAGCTGAAGTGGGACA R: AGCCATCAGGCTCGATGGCC	224	61
	ACTB	F: ACCCAGCACGATGAAGATCA R: GTAACGCAGCTAACAGTCCG	187	61
qPCR	*BMPR1B*	F: TGACGGACCTATACACCACA R: GTACCGAGGTCTGGCTTCTT	121	60
	BMP15	F: TGTTGGGCAAAAGCTCTGGA R: GCCATGCCACCAGAACTCAA	106	60
	GDF9	F: AACAGACGCCACCTCTACAA R: CACGATCCAGGTTAAACAGCA	124	60
	ACTB	F: CCAACCGTGAGAAGATGACC R: CCAGAGGCGTACAGGGACAG	97	60

https://doi.org/10.17221/101/2018-CJAS

reaction was carried out on a PCR instrument (Eppendorf, Germany). The cDNA quality was assessed by *ACTB* amplification, and the reverse transcription products were stored at –20°C.

Expression profiling and amplification system. Tissue-specific expression of *BMPR1B*, *BMP15*, and *GDF9* was investigated using transcription profiling, and *ACTB* was selected as the reference gene. The amplification reaction mixture volume of PCR was 20 µl and contained: 10 µl of 2× PCR Master Mix (Biomed, China), 0.5 µl of each forward and reverse primers (10 µM/l), 1.0 µl of cDNA, and 8 µl of ddH_2O. The amplification program was as follows: initial denaturation at 95°C for 5 min; denaturation at 95°C for 30 s, annealing temperature (see Table 1) for 30 s, and extension at 72°C for 60 s, optimization cycles (34, 36 and 36 cycles for *BMPR1B*, *BMP15* and *GDF9*, respectively), with a final extension at 72°C for 5 min, and then the PCR products were separated on 1.5% agarose gel.

qPCR system and program. The qPCR amplification reaction mixture (20 µl) contained 10 µl of SYBR® *Premix ExTaq*™ II (TaKaRa Bio Inc., China), 0.8 µl of each forward and reverse primer (10 µM/l), 2 µl of cDNA, and 6.4 µl of ddH_2O. The reaction carried out without template was used as a blank control. The program was carried out under the following conditions: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 5 s and 60°C for 30 s. After amplification, the melting curve was analysed. qPCR was performed in triplicate along with negative controls (H_2O as template) using the Roche LightCycler® 480 II (Roche, Switzerland). The gene expression data were normalized by the *ACTB* data.

Standard curve establishment. One microliter of cDNA from each sample was mixed. The homogenized cDNAs were diluted 1-, 2-, 4-, 8-, 16-, 32-, 64- and 128-fold, respectively, to obtain eight gradient concentrations. *BMPR1B*, *BMP15*, *GDF9*, and *ACTB* were quantified by qPCR using these diluted cDNAs as templates. The standard curves were plotted as Ct value against cDNA concentration (natural logarithm).

Statistical analysis. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001; Schmittgen and Livak 2008). Gene expression differences among different tissues and breeds were analysed by SPSS (Version 22.0, 2014) using a one-way analysis of variance. Multiple comparisons were performed using the least significant difference test.

RESULTS

Analysis of total RNA and cDNA. Total RNAs of 14 tissues were analysed using 1% agarose gel electrophoresis. Three bands (28S, 18S, and 5S) were observed. The optical density (OD) 260/280 nm ratios of the RNA samples were 1.8–2.0. These results revealed that the purity of the extracted total RNA of each sample was high without obvious contamination or degradation. In this study, 1 µg of each total RNA sample was used for the reverse transcription. After 5-fold dilution, the cDNA concentration of each sample for the PCR was about 240 ng/µl.

BMPR1B, BMP15, and GDF9 expression analysis. The expression profiling results showed that the fragment lengths of *BMPR1B*, *BMP15*, *GDF9*, and *ACTB* amplification products were consistent with the expected lengths (Figure 1).

As shown in Figure 1, *ACTB* was successfully amplified in all 14 tissues of the two sheep breeds, and expression levels were similar in these tissues. For STH and SNT sheep, *BMPR1B* was highly expressed in eight tissues (kidney, cerebrum, cerebellum, hypothalamus, pituitary, ovary, uterus, and oviduct). Furthermore, *BMPR1B* was also highly expressed in the adrenal tissue of SNT sheep. For STH sheep, *BMP15* was highly expressed in the ovary. For SNT sheep, *BMP15* was highly expressed in the ovary and oviduct. For both breeds, *GDF9* was expressed in all 14 tissues.

BMPR1B, BMP15, and GDF9 expression in the hypothalamic-pituitary-gonadal (HPG) axis. It is very difficult to quantitatively analyse gene expression using expression profiling. Therefore *BMPR1B*, *BMP15*, and *GDF9* expression in the HPG axis (hypothalamus, pituitary, and ovary), oviduct, and uterus in the STH and SNT sheep was analysed by qPCR.

As seen in Figure 2, in STH and SNT sheep, *BMPR1B* was expressed in five tissues, with the highest level in the ovary ($P < 0.01$). In STH sheep, *BMPR1B* expression was significantly lower in the hypothalamus and oviduct than in the ovary ($P < 0.01$), but significantly higher in the hypothalamus and oviduct than in the pituitary and uterus ($P < 0.01$). In SNT sheep, *BMPR1B* expression was significantly higher in the hypothalamus and ovary than in the pituitary, oviduct, and uterus ($P < 0.01$).

https://doi.org/10.17221/101/2018-CJAS

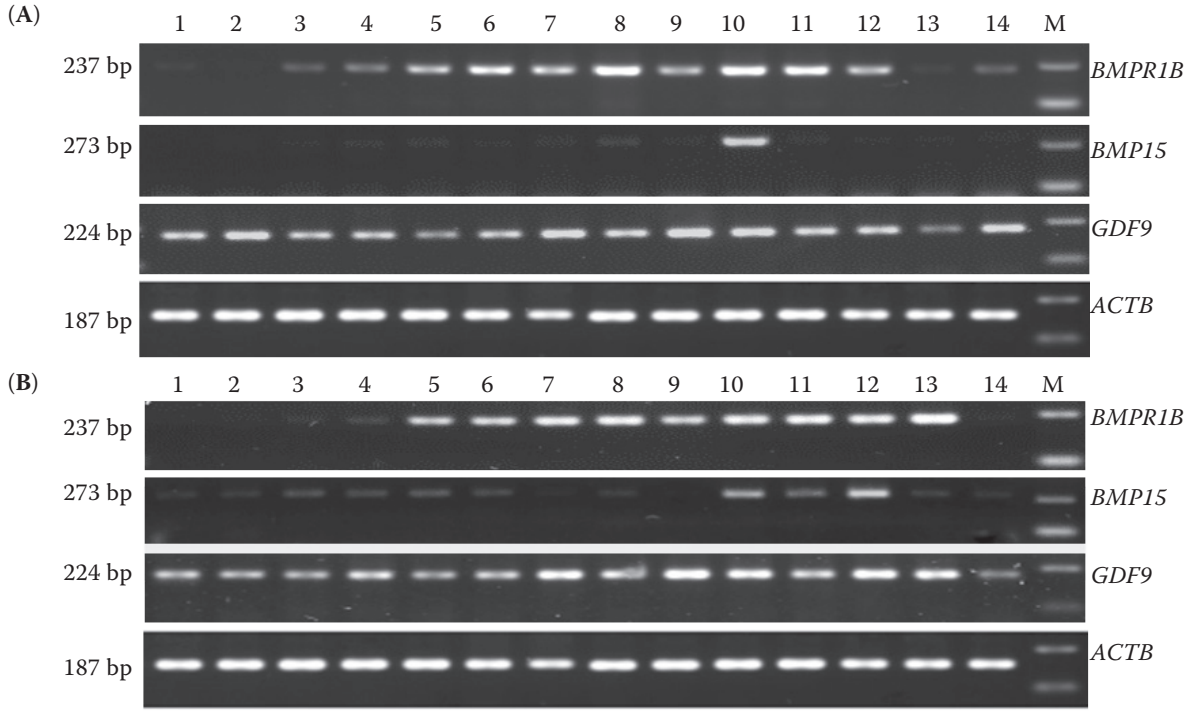

Figure 1. Tissue expression profiles of *BMPR1B*, *BMP15*, *GDF9*, and *ACTB* genes in (**A**) Small Tail Han sheep (STH) and (**B**) Sunite sheep (SNT) breeds

1–14: heart, liver, spleen, lung, kidney, cerebrum, cerebellum, hypothalamus, pituitary, ovary, uterus, oviduct, adrenal gland, and duodenum, respectively; M = DL2000 DNA marker

Compared with SNT sheep, *BMPR1B* expression levels of STH sheep were significantly higher in the ovary and oviduct ($P < 0.01$), but significantly lower in the pituitary ($P < 0.01$).

BMP15 expression was significantly higher in the ovary than in the other four tissues ($P < 0.01$). Between the two sheep breeds, *BMP15* expression was significantly lower in four tissues (pituitary, ovarian, oviduct, and uterine ones) of the STH sheep compared with the SNT sheep ($P < 0.01$). *GDF9* expression was significantly higher in the ovary than in the other four tissues in both breeds ($P < 0.01$). In STH sheep, gene expression was also significantly higher in the hypothalamus and pituitary compared with the oviduct and uterus ($P < 0.01$). Compared with SNT sheep, *GDF9* expression in STH sheep was significantly higher in the ovary and pituitary but lower in the uterus ($P < 0.01$).

DISCUSSION

BMPR1B function and expression. *BMPR1B* was the first identified major gene to exert a critical effect on Booroola Merino sheep litter size. As an important transmembrane receptor protein that is mainly involved in the TGFβ pathway, *BMPR1B* can regulate processes such as osteogenic differentiation, cell proliferation, and follicle development (Foroughinia et al. 2017). Previous research reported that *BMPR1B* was detected in brain, skeletal muscle, kidney, and reproductive tissues of Booroola sheep (Wilson et al. 2001). Subsequently, *BMPR1B* was found to be expressed in 10 tissues (hypothalamus, pituitary, ear, bone, kidney, skeletal muscle, ovary, uterus, spinal cord, and oviduct) of Chinese Merino sheep during the oestrus phase, when the gene expression was high in the ovary and ear (Yang et al. 2009).

In this study, *BMPR1B* was highly expressed in the kidney, brain, cerebellum, hypothalamus, pituitary, ovary, uterus, and oviduct; therefore *BMPR1B* may be associated with a wide range of biological effects because of its broad expression in these tissues. In addition, *BMPR1B* expression was significantly higher in the follicular tissue of prolific Hu sheep compared with non-prolific Hu sheep (Xu et al. 2010). In this study, *BMPR1B* expression was significantly higher in the ovary of STH sheep compared with SNT sheep; this

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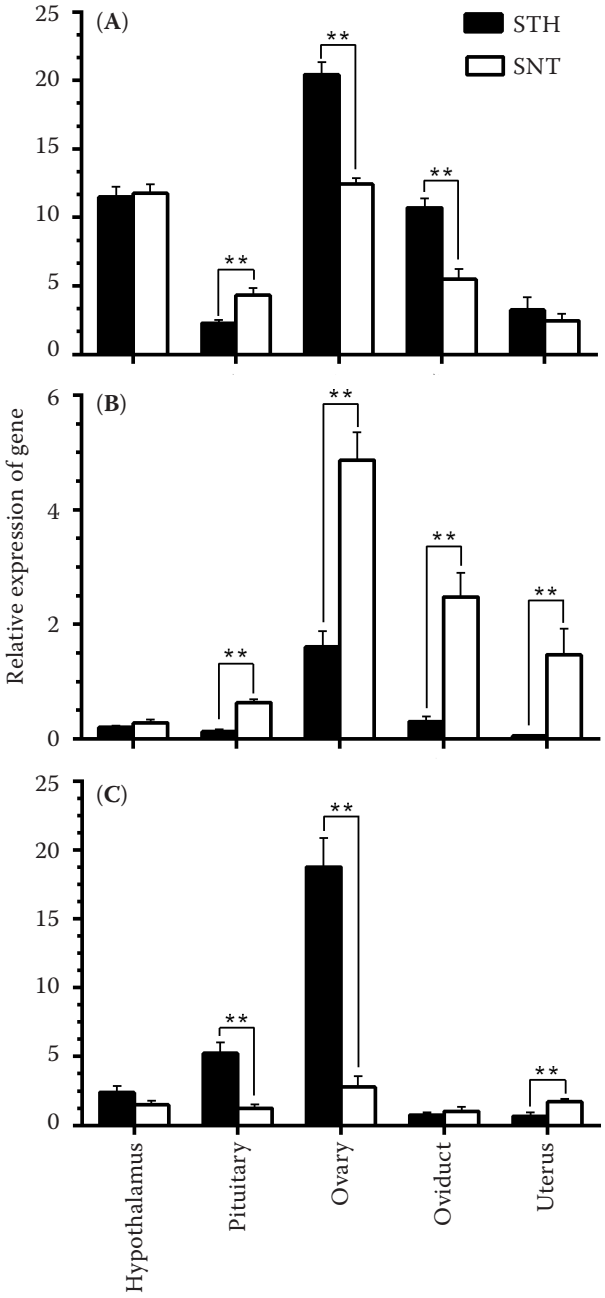

Figure 2. Comparison of *BMPR1B* (**A**), *BMP15* (**B**) and *GDF9* (**C**) genes expression in hypothalamus, pituitary, ovary, oviduct, and uterus of the Small Tail Han sheep (STH) and Sunite sheep (SNT) breeds

results are expressed as means ± standard error of the means; significant results: $^{**}P < 0.01$, $^{*}P < 0.05$

indicates that *BMPR1B* may be closely associated with high fecundity of sheep. However, whether high fecundity is due to the high expression or mutation of *BMPR1B* needs further research. Feary et al. (2007) found that *BMPR1B* expression differences affected the ovulation rate of Woodlands sheep with FecX2^{W} mutation, and BMPR1B may affect ovulation rate by regulating the BMP/Smad signalling pathway and related cytokines (Xu et al. 2010). It is well known that ovulation rate is one of the most important factors that affect sheep litter size. Therefore it can be speculated that high *BMPR1B* expression may affect ovarian function, and thus influence ovine fecundity.

BMP15 function and expression. Correlation studies showed that *BMP15*, both naturally mutated or knocked out in animal models, is a key gene that affects ovulation rate or litter size in mammals (Pramod et al. 2013). BMP15 can bind to type-I (e.g., BMPR1B) and type-II receptors on the granulosa/sheath cell membrane surrounding the oocyte (Shimasaki et al. 2004). Therefore, it plays pivotal regulatory roles in early oocyte growth and differentiation during normal follicular development. In this study, *BMP15* was found to be expressed in the ovary, pituitary, oviduct, and uterus of the two sheep breeds during the follicular phase. This result was inconsistent with those of previous studies which showed that *BMP15* was only specifically expressed in oocytes (Crawford et al. 2011). As for *BMP15* expression in the uterus, few studies were reported. Therefore in this study the result indicates that the functional diversity of *BMP15* may be involved in regulating the function of the uterus. BMP15 can downregulate the follicle-stimulating hormone (FSH) role by inhibiting the expression of the FSH receptor, promoting granulosa cell proliferation, and ensuring oocyte maturation (Otsuka and Shimasaki 2002). Therefore, higher *BMP15* expression was an important factor in maintaining the multiple follicular growth and development.

Moreover, the *BMP15* expression was lower in pituitary, ovarian, oviduct, and uterine tissues of STH sheep (high fecundity) compared with SNT sheep (low fecundity) ($P < 0.01$), which was consistent with previous findings in other sheep breeds (Dong et al. 2016). Compared with ewes with low prolificacy, the expression level of *BMP15* mRNA in oocytes of ewes with high prolificacy was significantly lower in Booroola Romney sheep, which may be responsible for the ovine high ovulation rate (Crawford et al. 2011). Otsuka et al. (2000) also found that BMP15 is a potent stimulator of GC proliferation. Cui et al. (2012) showed that BMP15 is secreted by the oocyte and acts on receptors (e.g. oestrogen, inhibin, and FSH receptors, etc.)

https://doi.org/10.17221/101/2018-CJAS

located on the granulosa cell (GC). Therefore, we speculated that the low *BMP15* expression may influence the proliferation of GC in individual follicles, resulting in relatively lower synthetic concentrations of FSH receptors. Ultimately, the follicle growth and development may be maintained under low FSH concentrations, which was the main reason for the increased ovulation in STH ewes.

In addition, we found that *BMP15* expression in the ovary was significantly lower in STH sheep compared with SNT sheep. However, it is still unclear whether this is a cause of the fecundity discrepancy. Prior literature showed that *BMP15* expression in the ovary of multiparous goat breeds was significantly higher than that of uniparous goat breeds (Pan et al. 2015). Therefore the inconsistent results may be due to species-related differences. Overall, the findings indicated that this gene plays an important role in the sheep ovary, because *BMP15* expression in the ovary was negatively correlated with lambing performance in sheep. However, *BMP15* expression in other tissues showed that there may also be other potential functions of this gene.

GDF9 function and expression. During follicular development, in addition to endocrine regulation of the HPG axis, GDF9 also performs an indispensable role in regulating follicular growth and differentiation, promoting granulosa cell proliferation, and maintaining follicular microenvironment stability (Vitt et al. 2000). *GDF9* expression was first studied in the human ovarian tissue (McPherron and Lee 1993); subsequently, it was detected in the ovaries of goat (Silva et al. 2005) and sheep (Feary et al. 2007). *GDF9* expression was observed in oocytes and other tissues, but varied among species (Pan et al. 2015).

This study revealed that *GDF9* was expressed in all detected tissues of the two sheep breeds, but it was most highly expressed in the ovary. In addition, *GDF9* expression was significantly higher in the ovary and pituitary of STH sheep compared with SNT sheep ($P < 0.01$). As an ovine candidate differentially expressed gene, *GDF*9 expression was significantly higher in the ovarian tissue of prolific Hu sheep compared with non-prolific Hu sheep ($P < 0.05$). Furthermore, another study reported that differential expression of *GDF9* may serve as an important basis for the high prolificacy of goat breeds, because its expression was significantly higher in the small antral follicle of polytocous Black Bengal goats compared with that of monotocous Sirohi goats (Pramod et al. 2013). However, *GDF9* expression did not significantly differ in the ovarian tissue of other goat breeds with different fecundity (Pan et al. 2015).

Vitt et al. (2000) found that GDF9 had a synergistic effect with FSH, BMP15, and other hormones or growth factors during the development of animal follicles. Therefore, *GDF9* expression may be associated with certain hormones or receptors in the pituitary, although this expression in other gonadal and non-gonadal tissues indicated that it may have more extensive biological functions. All of these findings showed that GDF9 may have substantial impacts on oestrous and reproductive traits in different sheep breeds, and high expression in the ovarian tissue showed that it may exert a pivotal role in improving the lambing performance and litter size of STH sheep.

CONCLUSION

This study revealed that *BMPR1B*, *BMP15*, and *GDF9* were highly expressed in the ovary of the two sheep breeds, and *BMPR1B* and *GDF9* expression was significantly higher in the ovary of STH sheep compared with SNT sheep ($P < 0.05$). However, *BMP15* expression was significantly lower in four tissues (pituitary, ovary, oviduct and uterus) of STH sheep compared with SNT sheep ($P < 0.01$). This research indicated that these three genes had important functions in the ovary and impacted reproductive performance. *BMPR1B* and *GDF9* expression may be positively correlated with litter size, whereas *BMP15* expression might be negatively correlated with litter size.

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