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Single nucleotide polymorphisms in *BMP2* and *BMP7* and the association with litter size in Small Tail Han sheep

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A B S T R A C T

Although it has been investigated for many years, the physiological processes regulating prolificacy in sheep remains unclear because of regulation by many genes. To better understand the effects of three single nucleotide polymorphisms (SNPs) comprising g.48462350C > T in *BMP2*, g.58171856C > G and g.58171886A > C in *BMP7*, a population genetic analysis was conducted using data obtained from genotyping in 768 sheep from six breeds (three polytocous and three monotocous). The results indicate that all the sheep breeds were considered to conform to the Hardy–Weinberg equilibrium ($P > 0.05$). The associations of these three SNPs with litter size in 384 Small Tail Han sheep were analyzed, therefore, and found to be correlated with fecundity as assessed by mean litter size ($P < 0.05$). Bioinformatic analysis indicated there was a transmembrane domain change that occurred after a mutation in BMP2 at g.48462350C > T, and changes involving transcription factors such as USF1, USF2 and INMS1 in the *BMP7* promoter region might be involved in greater sheep prolificacy.

1. Introduction

Sheep breeding, as an integral part of the animal industries in China and there are increasing revenues to farmers as a result of the abundance of sheep resources and popular sheep products. Even though there have been rapid advances in sheep breeding, the consumption of sheep products remains in short supply because of lack of understanding of the diverse physiological processes in sheep. Reproduction, one of most important processes in sheep husbandry, is important but extremely complicated, as it is controlled by numerous hormones (Zeydabadi Nejad et al., 2017; Stamatiades and Kaiser, 2018) released by the hypothalamus, pituitary and ovary. Normally, the female estrous cycle is initiated by the release of gonadotropin releasing hormone (GnRH) from the hypothalamus, which then functions in the pituitary and regulates the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH). These two hormones function in the ovary along with ovarian factors to promote follicular development (Juengel, 2018).

Results of numerous studies indicate environmental (Greives et al., 2007), nutritional (Forcada and Abecia, 2006), and genetic factors (McLaren, 1998) are important in regulation of sheep reproductive processes. The *FecB* gene, the first identified mutation to be significantly associated with sheep fecundity, was mapped to chromosome 6 (Montgomery et al., 1994). Results of subsequent studies (Souza et al., 2001; Wilson et al., 2001; Mulsant et al., 2001) indicate this mutation occurred in the gene encoding Bone

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Morphogenetic Protein receptor type 1 B (*BMPRIB*) at A764Gwith the result of the mutation being an amino acid change from glutamine to arginine. Furthermore, Fogarty (2009) investigated the relationship between the *FecB* mutation and ovulation rates, with results indicating the one copy of the *FecB* mutation being associated with the number of oocytes released with there being an increase of 1.26 and a litter size increase of 0.67. Furthermore, when there are two copies of this mutated gene, there is an increase of 3.61 and 0.77 in ovulation rate and litter size, respectively. In addition, other candidate genes such as *BMP15* and its mutations (Galloway et al., 2000; Bodin et al., 2007; Demars et al., 2013), and *GDF9* (McNatty et al., 2007) and its mutations (Våge et al., 2013) also have been associated with prolificacy of sheep. With an understanding of the associations of these mutations with increased prolificacy in sheep, there was a desire to gain further knowledge of how these genes and the mutations could affect sheep fecundity. The *BMP15* mutations were found to be associated with regulation of *GDF9* gene expression to regulate ovarian follicular development (Su et al., 2004), and the *BMPRIB* gene was also recognized to encode for a receptor for the protein resulting from *BMP15* gene expression (Moore et al., 2003). Recently developed strategies such as transcriptomics (Miao et al., 2015), proteomics (Miao et al., 2016) and metabolomics (Guo et al., 2018) were also applied to elucidate how the *FecB* mutation affects sheep reproduction. The detailed mechanisms of how these genes and mutations affect sheep reproduction, however, remain to be elucidated. When there are other genes or mutations associated with sheep fecundity or there are interactions in regulation of expression of candidate genes including *BMP15*, *GDF9, BMPRIB*, the identification of key genes or mutations affecting sheep fertility is an important aspect in understanding the underlying physiological changes that occur as a result of these mutations.

The *BMP2*, *BMP7*, like *BMP15*, *GDF9*, and *BMPRIB* genes encode for proteins of the transforming growth factor beta (TGF-β) superfamily (Shimasaki et al., 1999), and have important roles in signal transduction as a result of oligomerization of the BMP receptors BMPR-II, BMPRIA, and BMPRIB. These actions enable the phosphorylation of BMP receptor I, which in turn leads to the phosphorylation of Smad proteins, resulting in expression changes in target genes (Massagué, 1996; Miyazono et al., 2005). In one study, there was a report that an enhanced expression of the *BMP2* gene in the ovary of ewes can lead to an increase in estradiol (E2) concentrations *in vitro* (Souza et al., 2002). Juengel et al. (2006) reported that the expression of the *BMP2* gene in sheep can suppress progesterone concentrations. Interestingly, results of a study on human endometrial stromal cells also indicate that progesterone treatment can lead to a decrease in *BMP7* gene expression (Ako et al., 2010), and lead to greater numbers of FSH receptors and follicular development (Lee et al., 2004). Results of all these previous studies indicate the products from *BMP2* and *BMP7* gene expression have important functions in female reproduction. Whether the mutations of the *BMP2*, *BMP7* genes affect sheep prolificacy similar to what occurs as a result of mutations in the *BMPRIB, BMP15, GDF9* genes remains to be elucidated. The aim of the present study was to enhance understanding of the effect of these mutations on reproductive functions.

2. Materials and methods

2.1. Ethics statement and sample collection

All animal used in this study was approved by the Science Research Department (in charge of animal welfare issues) of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (IAS-CAAS; Beijing, P. R. China). In addition, there was ethics approval by the animal ethics committee of IAS-CAAS (No. IASCAAS-AE-03, 12 December 2016).

Jugular blood samples from 768 sheep of six breeds were collected into EDTA-coated tubes and stored at −20 °C until used for DNA isolation. Among the sheep breeds included in the study, the Small Tail Han (n = 384), Hu sheep (n = 128), and Cele Black (n = 52) sheep are the greater fecundity breeds, and the Sunite (n = 21), Prairie Tibetan (n = 161), and Tan (n = 22) sheep are the lesser fecundity breeds.

2.2. DNA isolation and genotyping the known SNPs in BMP2 and BMP7

Genomic DNA was isolated using blood genomic DNA extraction kits (TIANGEN Biotech Co., Ltd., Beijing, P. R. China). The single-base extended primers used for detecting known SNPs including g.48462350C > T, g.58171856C > G and g.58171886A > C, and polymerase chain reaction (PCR) procedures were designed using the MassARRAY Assay Design v. 3.1 according to sheep BMP2 and BMP7 sequences available in the NCBI GenBank (accession numbers: XM_012156016, NM_001308564). The MassARRAY (Johansen et al., 2013) was subsequently applied to genotype the genomic DNA of all 768 sheep. The MassARRAY is a recently developed strategy used to detect SNPs. Amplification of the mutation sequences was initially conducted. Single-base extended PCR reactions were subsequently conducted using specific primers. The molecular weight of products after extension differs because of different bases at mutation sites. This difference can be detected through time-of-flight mass spectrometry, resulting in separation of the products resulting because of the different genotypes.

2.3. Statistical analysis

Allele frequencies, genotype frequencies, P values, polymorphism information content (PIC), heterozygosity (HE) and number of effective alleles (NE) were calculated using the data obtained from genotyping results. Sheep populations with $P > 0.05$ (Chi-Square Test) were considered to conform to the Hardy–Weinberg equilibrium (Ortega et al., 2016). A linear model using the equation $y_{ijn} = \mu + P_i + G_j + I_{PG} + e_{ijn}$ was applied subsequently to analyze the association of genotypes and litter size, in which y_{ijn} represents phenotypic value (litter size); μ is the population mean; P_j shows the fixed effect of the ith parity (i = 1, 2, or 3); G_j represents the effect of the jth genotypes (j = 1, 2, or 3); I_{PG} represents the interactive effect of parity and genotype; and e_{ijn} represents random error.

Table 1
SNP information for *BMP2* and *BMP7*.

Gene	SNP position	Location	Consequence type	Nucleotide change	Codon change	Amino acid change
BMP2	g.48462350	Coding region	missense	C > T	GCG/GTG	A/V
BMP7	g.58171856	Promoter region	missense	C > G	–	–
	g.58171886	Promoter region	missense	A > C	–	–

Importantly, statistical analyses were conducted using SAS (V. 8.1) (SAS Institute Inc., Cary, NC, USA), and the least significant difference test was used to evaluate differences between groups. The $P < 0.05$ value was considered to indicate significance.

2.4. Bioinformatics analysis

First, the integral and coding sequences of *BMP2*, *BMP7* were obtained from NCBI (https://www.ncbi.nlm.nih.gov/), and amino acid sequences were subsequently obtained from UniProt (https://www.uniprot.org/). The transmembrane domains before and after mutations in *BMP2* were predicted using TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Prediction of the secondary structure of *BMP2* and its mutants occurred using PredictProtein (https://www.predictprotein.org/). In addition, the promoter sequence of *BMP7* was obtained from UCSC Table Browser (https://genome.ucsc.edu/cgi-bin/hgTables). The structures of transcriptional factor binding sites before and after mutations in promoter regions were also evaluated using JASPAR (http://jaspar.binf.ku.dk/cgi-bin/jaspar_db.pl).

3. Results

3.1. Population genetic analysis of known SNPs in BMP2 and BMP7

Basic information on these known SNPs is listed in Table 1. Results from population genetics analysis (Table 2) indicated that the three genotypes g.48462350C > T including the base pairs CC, CT and TT, g.58171856C > G including CC, CG and GG, and g.58171886A > C including AA, AC and CC were all detected. Most sheep breeds at the g.48462350C > T locus, but not Prairie Tibetan sheep, were moderately polymorphic ($0.25 < \text{PIC} < 0.5$). For the g.58171856C > G and g.58171886A > C loci, however, genotyping results indicated that all the sheep breeds had a relatively lesser polymorphism ($\text{PIC} < 0.05$). In addition, it is important to note that all the sheep breeds conformed to the Hardy–Weinberg equilibrium ($P > 0.05$).

3.2. Association analysis of three SNPs with litter size in Small Tail Han sheep

To better understand the association of g.48462350C > T, g.58171856C > G and g.58171886A > C with litter size, an association analysis was performed for these three SNPs in terms of litter size in 384 Small Tail Han sheep (Table 3). The litter size for each genotype increased from the first to the third parity. Furthermore, the g.48462350C > T SNP was correlated with litter size at

Table 2
Population genetic analysis of g.48462350C > T, g.58171856C > G, and g.58171886A > C in six sheep breeds.

Locus	Breed	Genotype Frequency			Allele Frequency		PIC	HE	NE	*Chi*-Square Test(*P*-value)
g.48462350C > T		CC	CT	TT	C	T				
	Small Tail Han sheep	0.46	0.43	0.11	0.67	0.33	0.34	0.44	1.79	0.58
	Prairie Tibetan sheep	0.98	0.02	0.00	0.99	0.01	0.02	0.02	1.02	0.87
	Hu sheep	0.19	0.44	0.37	0.41	0.59	0.37	0.48	0.19	0.37
	Cele black sheep	0.27	0.61	0.12	0.58	0.42	0.37	0.49	1.95	0.06
	Sunite sheep	0.24	0.52	0.24	0.50	0.50	0.38	0.50	2.00	0.83
	Tan sheep	0.09	0.32	0.59	0.25	0.75	0.30	0.38	1.60	0.48
g.58171856C > G		CC	CG	GG	C	G				
	Small Tail Han sheep	0.89	0.10	0.01	0.95	0.05	0.10	0.10	1.11	0.36
	Prairie Tibetan sheep	0.92	0.07	0.01	0.96	0.04	0.08	0.08	1.09	0.19
	Hu sheep	0.96	0.04	0.00	0.98	0.02	0.04	0.04	0.96	0.82
	Cele black sheep	0.98	0.02	0.00	0.99	0.01	0.02	0.02	1.02	0.94
	Sunite sheep	0.95	0.05	0.00	0.98	0.02	0.05	0.05	1.05	0.91
	Tan sheep	0.91	0.09	0.00	0.95	0.05	0.08	0.09	1.10	0.82
g.58171886A > C		AA	AC	CC	A	C				
	Small Tail Han sheep	0.82	0.16	0.02	0.90	0.10	0.17	0.18	1.22	0.08
	Prairie Tibetan sheep	0.96	0.04	0.00	0.98	0.02	0.04	0.04	1.04	0.78
	Hu sheep	0.93	0.07	0.00	0.96	0.04	0.07	0.07	1.07	0.68
	Cele black sheep	0.96	0.04	0.00	0.98	0.02	0.04	0.04	1.04	0.89
	Sunite sheep	0.90	0.10	0.00	0.95	0.05	0.09	0.09	1.10	0.82
	Tan sheep	0.68	0.32	0.00	0.84	0.16	0.23	0.27	1.37	0.37

Table 3
Least-square means and standard errors of litter size in Small Tail Han sheep in different parities for each genotype.

Locus	Genotype	Litter Size			
		First Parity (N)	Second Parity (N)	Third Parity (N)	Total (N)
g.48462350C > T	CC	2.10(154) ± 0.068	2.24(149) ± 0.074	2.72(61) ± 0.138^{a}	2.26(364) ± 0.049^{a}
	CT	2.26(137) ± 0.072	2.45(128) ± 0.08	2.78(49) ± 0.154^{a}	2.42(314) ± 0.053^{ab}
	TT	2.21(38) ± 0.136	2.42(36) ± 0.151	3.40(15) ± 0.279^{b}	2.50(89) ± 0.099^{b}
g.58171856C > G	CC	2.15(317) ± 0.047	2.31(302) ± 0.052^{a}	2.80(117) ± 0.1	2.32(736) ± 0.034^{a}
	GC	2.15(2) ± 0.146	2.40(2) ± 0.164^{a}	3.00(2) ± 0.312	2.39(6) ± 0.108^{a}
	GG	3.00(33) ± 0.593	3.50(30) ± 0.637^{b}	3.50(12) ± 0.764	3.33(75) ± 0.038^{b}
g.58171886A > C	AA	2.13(279) ± 0.05^{a}	2.32(263) ± 0.056	2.83(102) ± 0.106	2.31(644) ± 0.037^{a}
	CA	2.10(12) ± 0.116^{a}	2.33(11) ± 0.126	2.68(6) ± 0.227	2.29(29) ± 0.083^{a}
	CC	2.75(52) ± 0.242^{b}	2.73(52) ± 0.274	3.50(22) ± 0.435	2.90(126) ± 0.173^{b}

Note: Different letters for the groups indicate differences ($P < 0.05$).

the third parity, and sheep with the TT variant had the largest litters ($P < 0.05$). The g.58171856C > G SNP was correlated with litter size at the second parity, and sheep with the GG variant had the largest litters ($P < 0.05$). The g.58171886A > C SNP was correlated with litter size at first parity, and sheep with the GG variant had the largest litters ($P < 0.05$). Importantly, the mean litter size of homozygous mutants including the TT, GG, and CC variants in three SNPs were larger than for the other two genotypes ($P < 0.05$).

3.3. Bioinformatics analysis of BMP2

To analyze the changes before and after the mutation g.58171856C > G, a transmembrane domain prediction using the amino acid (AA) sequence was applied. The results indicated that a mutation from C to G could lead to the presence of a unique transmembrane domain (Fig. 1). This significant change could lead to functional changes in the encoded protein. In addition, prediction of the protein secondary structure before and after the mutation at g.48462350C > T using the AA sequence was also performed. Compared with the wild-type genotype, the results indicated that there was one protein-binding region in proximity to AA number 8, while there as a DNA-binding region in proximity to AA 10, and one protein-binding region in proximity to AA 275 that were no

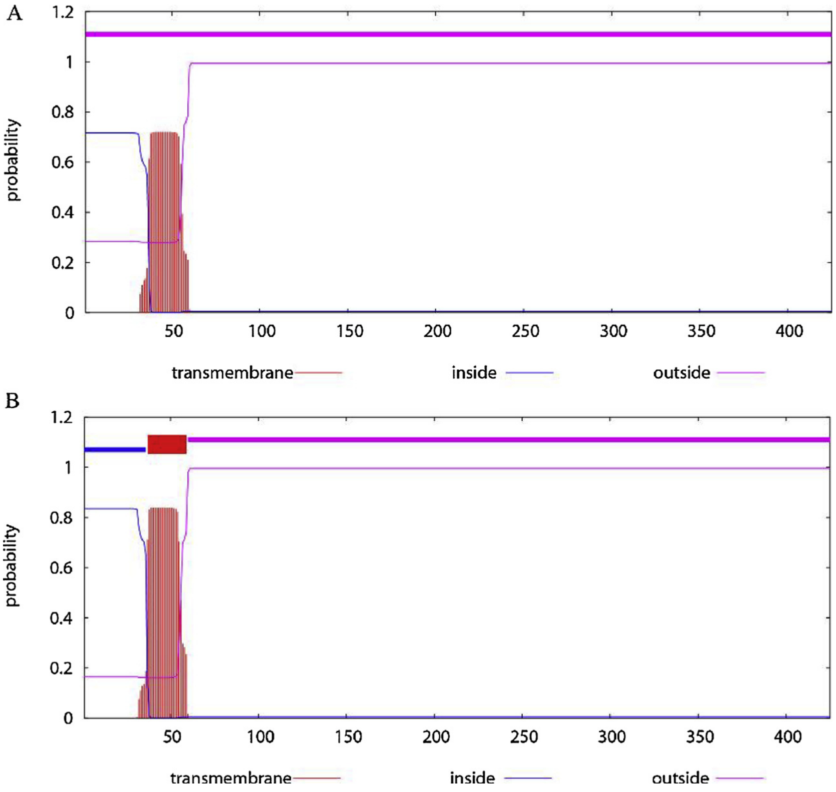


Fig. 1. Transmembrane domain of the protein encoded by *BMP2* before and after mutation at g.48462350C > T predicted using the amino acid sequence as reported in TMHMM; (A) The transmembrane domain before mutation; (B) The transmembrane domain after mutation.

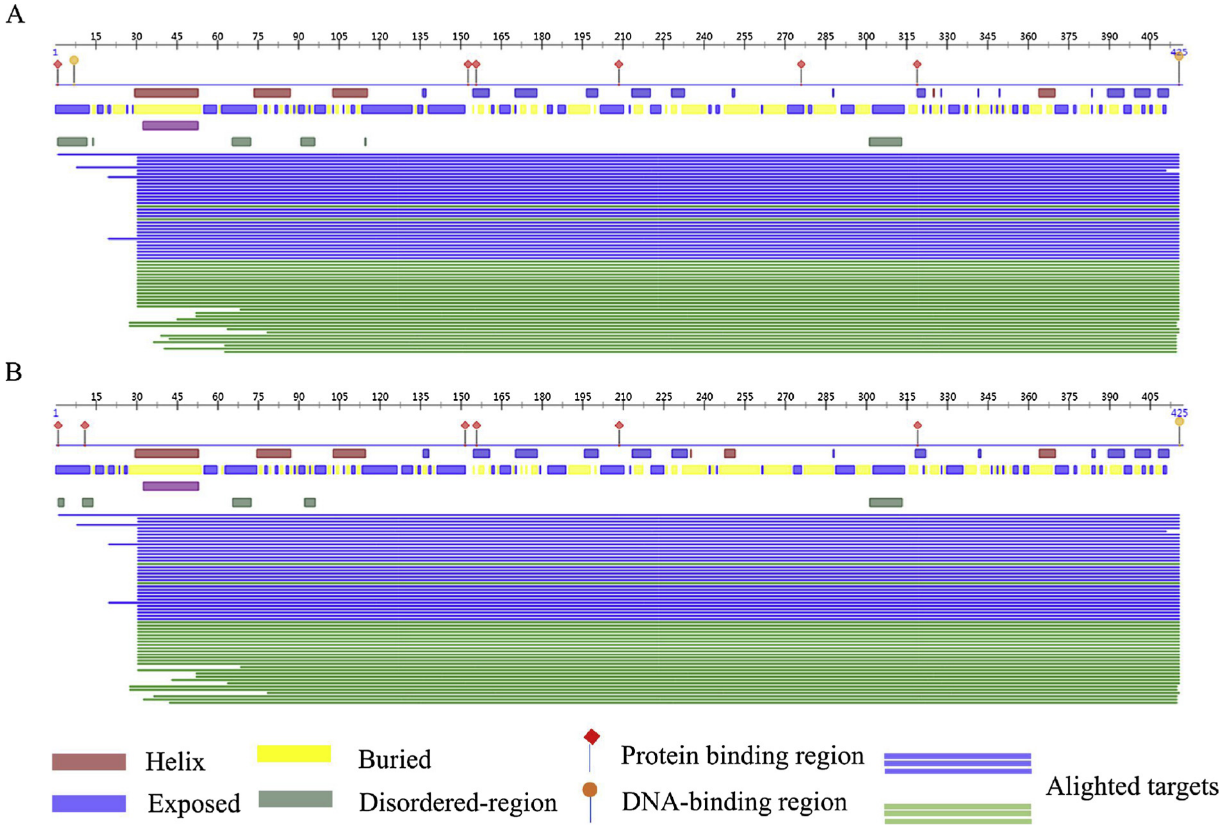


Fig. 2. Secondary protein structure for the *BMP2* product before and after the mutation at g.48462350C > T based on its predicted amino acid sequence; (A) Secondary protein structure before the mutation; (B) Secondary protein structure after the mutation.

longer present as a result of this mutation (Fig. 2).

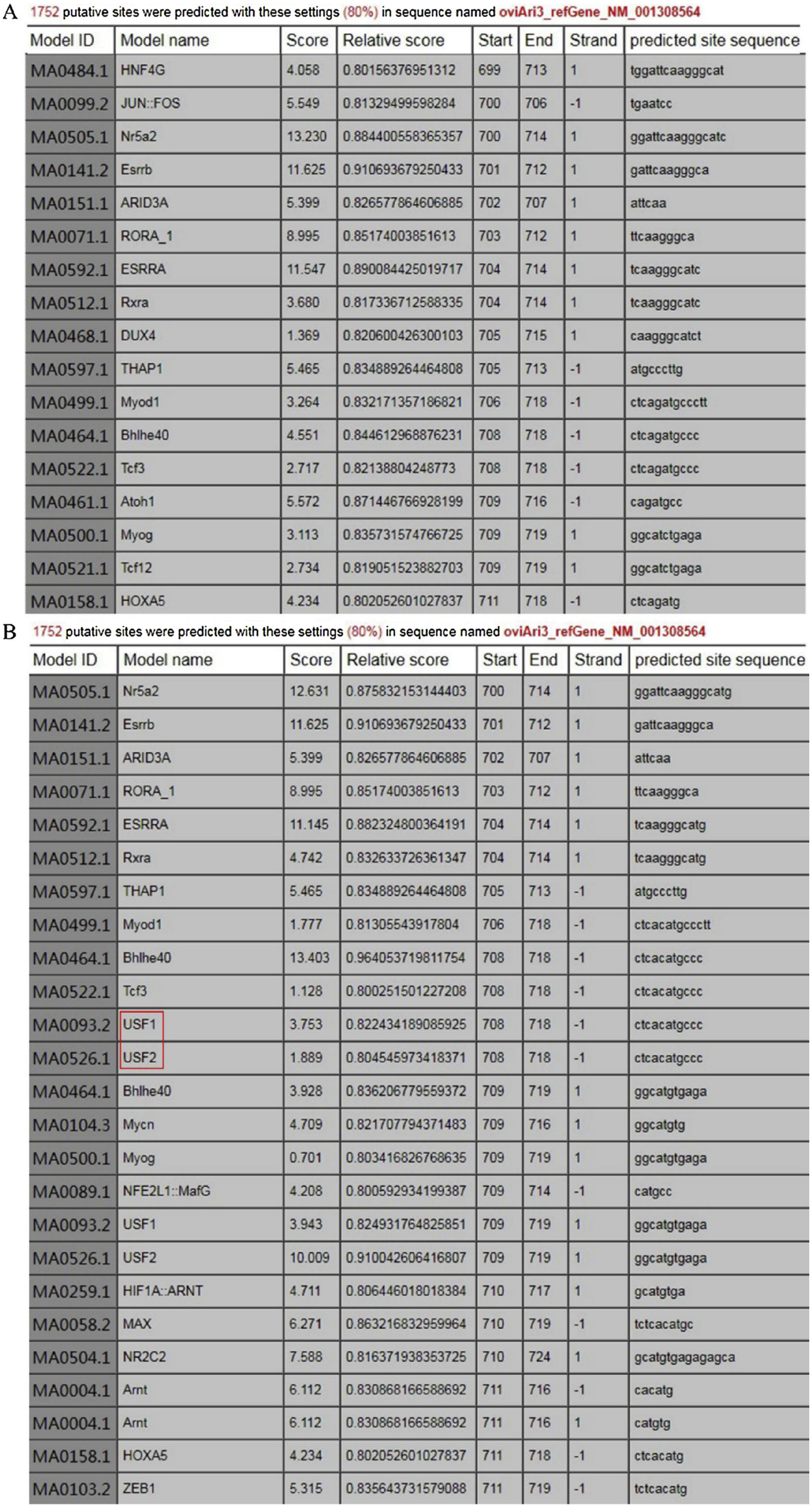
3.4. Bioinformatics analysis of BMP7

Regarding *BMP7,* there was a primary focus on its promoter region, as the mutations containing g.58171856C > G and g.58171886A > C occurred at promoter regions. The promoter sequence obtained from UCSC Table Browser was used to predict transcriptional factors (TFs). The g.58171856C > G SNP (Fig. 3) was predicted to be involved in many TF-binding regions such as transcription factor activator protein-2A (TFAP2A), and transcription factor activator protein-2C (TFAP2C) for which scores were greater than 11. In addition, many TFs, such as the upstream transcription factor 1 (USF1) and upstream transcription factor 2 (USF2) also were present with the g.58171856C > G. SNP for the g.58171886A > C SNP, however, there was an absence of several TFs such as insulinoma-associated 1 (INMS1) as a result of this mutation (Fig. 4).

4. Discussion

The *BMP2* and *BMP7* are two important genes encoding the TGF-β super-family that have important functions in sheep female reproduction (Rajesh et al., 2018; da Cunha et al., 2017; Zhang et al., 2017). To understand whether *BMP2* and *BMP7* could affect sheep reproduction, there was previous studies focused on the expression of these genes in the Small Tail Han (polytocous breed) and Sunite (monotocous breed) breeds in seven tissues including the brain, cerebellum, hypothalamus, pituitary, uterus, ovary, and oviduct (Zhang et al., 2018). The relative expression of the *BMP2* gene in the hypothalamus, oviduct, ovary, pituitary, and cerebellum of Small Tail Han sheep was greater than in Sunite sheep. Furthermore the expression of the *BMP7* gene in the pituitary, brain, hypothalamus, oviduct, and ovary of Small Tail Han sheep was greater than in Sunite sheep. In addition, Zhang et al. (2017) reported that *BMP7* is a potential factor involved in sheep prolificacy as a result of findings using the whole genome DNA methylation analysis. Rajesh et al. (2018) reported that both *BMP2* and *BMP7* gene expression can increase estradiol production and enhance the survival of ovarian granulosa cells. Besides, considering the important roles of the hypothalamus, pituitary, uterus, and ovary in female reproduction and the association analysis of different genotypes in the present study with litter size in Small Tail Han sheep, results indicate that *BMP2* and *BMP7* are two important candidate genes affecting ewe prolificacy.

Transmembrane domains of cells are integral components enabling numerous proteins to function (Nadir et al., 2012; Jang et al.,



A 1752 putative sites were predicted with these settings (80%) in sequence named oviAri3_refGene_NM_001308564

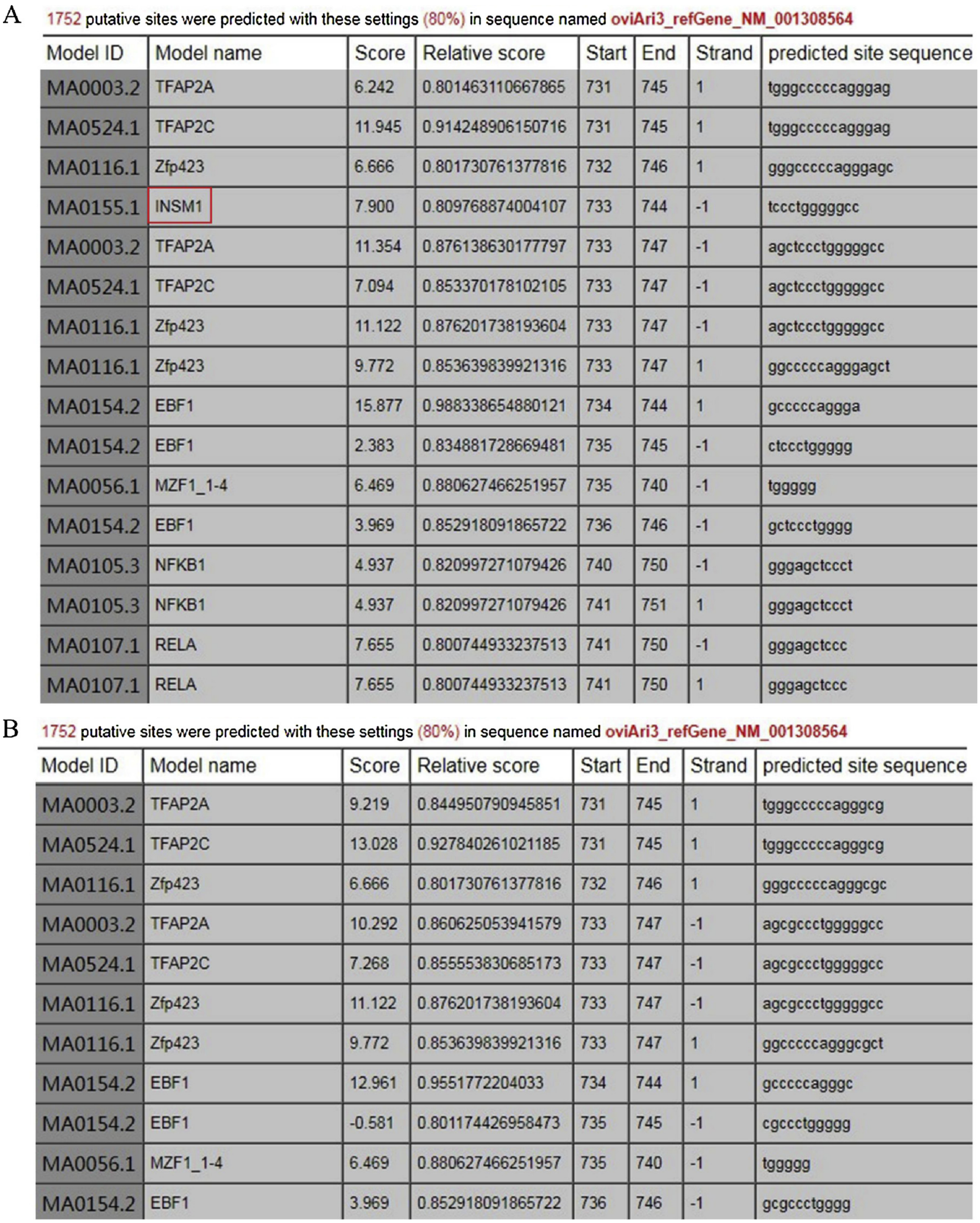
Model ID	Model name	Score	Relative score	Start	End	Strand	predicted site sequence
MA0484.1	HNF4G	4.058	0.80156376951312	699	713	1	tggattcaagggcat
MA0099.2	JUN::FOS	5.549	0.81329499598284	700	706	-1	tgaatcc
MA0505.1	Nr5a2	13.230	0.884400558365357	700	714	1	ggattcaagggcatc
MA0141.2	Esrrb	11.625	0.910693679250433	701	712	1	gattcaagggca
MA0151.1	ARID3A	5.399	0.826577864606885	702	707	1	attcaa
MA0071.1	RORA_1	8.995	0.85174003851613	703	712	1	ttcaagggca
MA0592.1	ESRRA	11.547	0.890084425019717	704	714	1	tcaagggcatc
MA0512.1	Rxra	3.680	0.817336712588335	704	714	1	tcaagggcatc
MA0468.1	DUX4	1.369	0.820600426300103	705	715	1	caagggcatct
MA0597.1	THAP1	5.465	0.834889264464808	705	713	-1	atgcccttg
MA0499.1	Myod1	3.264	0.832171357186821	706	718	-1	ctcagatgccctt
MA0464.1	Bhlhe40	4.551	0.844612968876231	708	718	-1	ctcagatgccc
MA0522.1	Tcf3	2.717	0.82138804248773	708	718	-1	ctcagatgccc
MA0461.1	Atoh1	5.572	0.871446766928199	709	716	-1	cagatgcc
MA0500.1	Myog	3.113	0.835731574766725	709	719	1	ggcatctgaga
MA0521.1	Tcf12	2.734	0.819051523882703	709	719	1	ggcatctgaga
MA0158.1	HOXA5	4.234	0.802052601027837	711	718	-1	ctcagatg

B 1752 putative sites were predicted with these settings (80%) in sequence named oviAri3_refGene_NM_001308564

Model ID	Model name	Score	Relative score	Start	End	Strand	predicted site sequence
MA0505.1	Nr5a2	12.631	0.875832153144403	700	714	1	ggattcaagggcatg
MA0141.2	Esrrb	11.625	0.910693679250433	701	712	1	gattcaagggca
MA0151.1	ARID3A	5.399	0.826577864606885	702	707	1	attcaa
MA0071.1	RORA_1	8.995	0.85174003851613	703	712	1	ttcaagggca
MA0592.1	ESRRA	11.145	0.882324800364191	704	714	1	tcaagggcatg
MA0512.1	Rxra	4.742	0.832633726361347	704	714	1	tcaagggcatg
MA0597.1	THAP1	5.465	0.834889264464808	705	713	-1	atgcccttg
MA0499.1	Myod1	1.777	0.81305543917804	706	718	-1	ctcacatgccctt
MA0464.1	Bhlhe40	13.403	0.964053719811754	708	718	-1	ctcacatgccc
MA0522.1	Tcf3	1.128	0.800251501227208	708	718	-1	ctcacatgccc
MA0093.2	USF1	3.753	0.822434189085925	708	718	-1	ctcacatgccc
MA0526.1	USF2	1.889	0.804545973418371	708	718	-1	ctcacatgccc
MA0464.1	Bhlhe40	3.928	0.836206779559372	709	719	1	ggcatgtgaga
MA0104.3	Mycn	4.709	0.821707794371483	709	716	1	ggcatgtg
MA0500.1	Myog	0.701	0.803416826768635	709	719	1	ggcatgtgaga
MA0089.1	NFE2L1::MafG	4.208	0.800592934199387	709	714	-1	catgcc
MA0093.2	USF1	3.943	0.824931764825851	709	719	1	ggcatgtgaga
MA0526.1	USF2	10.009	0.910042606416807	709	719	1	ggcatgtgaga
MA0259.1	HIF1A::ARNT	4.711	0.806446018018384	710	717	1	gcatgtga
MA0058.2	MAX	6.271	0.863216832959964	710	719	-1	tctcacatgc
MA0504.1	NR2C2	7.588	0.816371938353725	710	724	1	gcatgtgagagagca
MA0004.1	Arnt	6.112	0.830868166588692	711	716	-1	cacatg
MA0004.1	Arnt	6.112	0.830868166588692	711	716	1	catgtg
MA0158.1	HOXA5	4.234	0.802052601027837	711	718	-1	ctcacatg
MA0103.2	ZEB1	5.315	0.835643731579088	711	719	-1	tctcacatg

(caption on next page)

Fig. 3. Transcriptional factors-binding locus prediction of BMP7 before and after mutation at g.58171856C > G using promoter region by JASPAR; (A) Transcriptional factor binding-region before mutation; (B) Transcriptional factor-binding region after mutation.



A

1752 putative sites were predicted with these settings (80%) in sequence named oviAri3_refGene_NM_001308564

Model ID	Model name	Score	Relative score	Start	End	Strand	predicted site sequence
MA0003.2	TFAP2A	6.242	0.801463110667865	731	745	1	tgggcccccagggag
MA0524.1	TFAP2C	11.945	0.914248906150716	731	745	1	tgggcccccagggag
MA0116.1	Zfp423	6.666	0.801730761377816	732	746	1	gggcccccagggagc
MA0155.1	INSM1	7.900	0.809768874004107	733	744	-1	tccctgggggcc
MA0003.2	TFAP2A	11.354	0.876138630177797	733	747	-1	agctccctgggggcc
MA0524.1	TFAP2C	7.094	0.853370178102105	733	747	-1	agctccctgggggcc
MA0116.1	Zfp423	11.122	0.876201738193604	733	747	-1	agctccctgggggcc
MA0116.1	Zfp423	9.772	0.853639839921316	733	747	1	ggcccccagggagct
MA0154.2	EBF1	15.877	0.988338654880121	734	744	1	gcccccaggga
MA0154.2	EBF1	2.383	0.834881728669481	735	745	-1	ctccctggggg
MA0056.1	MZF1_1-4	6.469	0.880627466251957	735	740	-1	tggggg
MA0154.2	EBF1	3.969	0.852918091865722	736	746	-1	gctccctgggg
MA0105.3	NFKB1	4.937	0.820997271079426	740	750	-1	gggagctccct
MA0105.3	NFKB1	4.937	0.820997271079426	741	751	1	gggagctccct
MA0107.1	RELA	7.655	0.800744933237513	741	750	-1	gggagctccc
MA0107.1	RELA	7.655	0.800744933237513	741	750	1	gggagctccc

B

1752 putative sites were predicted with these settings (80%) in sequence named oviAri3_refGene_NM_001308564

Model ID	Model name	Score	Relative score	Start	End	Strand	predicted site sequence
MA0003.2	TFAP2A	9.219	0.844950790945851	731	745	1	tgggcccccagggcg
MA0524.1	TFAP2C	13.028	0.927840261021185	731	745	1	tgggcccccagggcg
MA0116.1	Zfp423	6.666	0.801730761377816	732	746	1	gggcccccagggcgc
MA0003.2	TFAP2A	10.292	0.860625053941579	733	747	-1	agcgccctgggggcc
MA0524.1	TFAP2C	7.268	0.855553830685173	733	747	-1	agcgccctgggggcc
MA0116.1	Zfp423	11.122	0.876201738193604	733	747	-1	agcgccctgggggcc
MA0116.1	Zfp423	9.772	0.853639839921316	733	747	1	ggcccccagggcgct
MA0154.2	EBF1	12.961	0.9551772204033	734	744	1	gcccccagggc
MA0154.2	EBF1	-0.581	0.801174426958473	735	745	-1	cgccctggggg
MA0056.1	MZF1_1-4	6.469	0.880627466251957	735	740	-1	tggggg
MA0154.2	EBF1	3.969	0.852918091865722	736	746	-1	gcgccctgggg

Fig. 4. Transcriptional factors-binding locus prediction of *BMP7* before and after mutation at g.58171886A > C using promoter region by JASPAR, where the binding possibility increases with the increase of score; (A) Transcriptional factor-binding region before mutation; (B) Transcriptional factor-binding region after mutation.

2018; Guna and Hegde, 2018). Shacklett et al. (1998) reported that the transmembrane domain is necessary for the envelope protein to function, which affects many processes such as neuro-pathogenesis (Hansen et al., 2017). Xiao et al. (2008) reported that the transmembrane domain 5 functions as a modulator for menthol sensitivity of TRPA1 channels in mammals. In addition, Tarasova et al. (1999) reported that disruptions involving transmembrane domain interactions resulted in dysfunction in G-protein-coupled receptors. Transmembrane domains in specific cells can also affect reproduction. Thus, Muramatsu and Miyauchi (2003) suggested that Basigin, as a transmembrane protein, affects the reproductive system of mice in which a deficiency of the encoding gene resulted in the mice being sterile and having neurological abnormalities. Bramble et al. (2016) reported that two mutations in the transmembrane domain of the FSH receptor can cause primary ovarian failure. The BMP2, is a non-transmembrane protein before a mutation occurs, however, after the mutation functions as a transmembrane protein when the mutation is from C to T at g.48462350. Furthermore, considering the finding in the present study of the association of the g.48462350C > T polymorphism with litter size in Small Tail Han sheep, there is the possibility that this transmembrane domain might be responsible, at least in part, for the differences in litter sizes between polytocous and monotocous sheep.

The TFs include many proteins which function to modulate gene expression transcriptional processes and phenotypes directly (Francois et al., 2018). The USF2, as a TF, with a score of greater than 10 with the JASPAR predication, might bind to the region where a single base mutation (g.58171856C > G) occurred. Luo and Sawadogo (1996) reported that USF2, as one of the functional domains in the promoter region, can induce transcriptional activation as indicated by results of immunofluorescence studies. Furthermore, the interaction of USF2 with other TFs, such as USF1, which is also predicted to be a TF, can enhance the expression of particular genes such as *FMR1* (Kumari and Usdin, 2001). There has been identification of an SNP in the *FOXE1* gene with this SNP being integral in determining the function of *FOXE1* by enhancing interactions of USF1 and USF2 (Ruiz-llorente et al., 2009). With the TF prediction in the present study using the JASPAR predication, two important TFs, USF1 and USF2, were predicted to bind to the regions containing SNPs. Considering this occurrence in the three genotypes in g.58171856C > G and associations with litter size in Small Tail Han sheep, it is speculated that the recruitment of USF1 and USF2 might result in differential expression of the *BMP7* gene in this breed, which might account for the increased litter size.

The TFAP2A, as a key TF, has pivotal actions on transcription, additionally, the probability (JASPAR predication score) of binding to TFAP2A increased along with the change from a normal to a mutant genotype. Koinuma et al. (2009) identified Smad2/3 binding sites in the promoter regions of numerous genes where there was also enrichment of TFAP2A. In addition, TFAP2A could also modulate Smad2/3 binding sites in the promoter region of the *CDKN1A* gene as indicated by results from chromatin immunoprecipitation studies. The Smad2/3 binding sites, whether in normal or mutant *BMP7* promoter regions, however were not detected, which might indicate there are species-specific differences. Other interactive associations with proteins remain to be elucidated. It is important to note that there are many TFs that are no longer present when there is mutation at g.58171886A > C. The INMS1, as one of these, can inhibit the promoter region of the neuroD/beta2 gene by recruiting cyclin D1 and other factors (Liu et al., 2006). Importantly, in the present study there was not detection of this TF because of the mutation (g.58171886A > C) at its promoter region, which means the inhibitory effects might be, in part, abolished because the other TFs such as INMS1 were no longer present that result in enhanced expression of the *BMP7* gene, leading to a further enhancement of sheep fecundity.

5. Conclusions

In summary, three SNPs containing g.48462350C > T in *BMP2*, g.58171856C > G and g.58171886A > C in *BMP7* were associated with increased litter size in Small Tail Han sheep ($P < 0.05$). In addition, bioinformatic analysis indicated the appearance of a transmembrane domain after the g.48462350C > T mutation in *BMP2*. In addition, the changes in TFs such as USF1, USF2 and INMS1 in the *BMP7* promoter region might account for increased sheep prolificacy. Findings in the present study indicate that *BMP2* and *BMP7* might serve as candidate genes affecting litter size in sheep.

Conflicts of interest

The authors declare that they have no competing interests.

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