



Genome-Wide Association Analyses Highlight the Potential for Different Genetic Mechanisms for Litter Size Among Sheep Breeds

Song-Song Xu^{1,2†}, Lei Gao^{3,4†}, Xing-Long Xie^{1,2}, Yan-Ling Ren⁵, Zhi-Qiang Shen⁵, Feng Wang⁶, Min Shen^{3,4}, Emma Eyþórsdóttir⁷, Jón H. Hallsson⁷, Tatyana Kiseleva⁸, Juha Kantanen⁹ and Meng-Hua Li^{1,2*}

OPEN ACCESS

Edited by:

Joram Mwashigadi Mwacharo,
International Center for Agricultural
Research in the Dry Areas (ICARDA),
Ethiopia

Reviewed by:

Shahin Eghbalsaied,
Islamic Azad University, Iran
Clare A. Gill,
Texas A&M University, United States
David Wragg,
The University of Edinburgh,
United Kingdom
Mourad Rekik,
International Center for Agricultural
Research in the Dry Areas (ICARDA),
Jordan

*Correspondence:

Meng-Hua Li
menghua.li@ioz.ac.cn

†These authors have contributed
equally to this work.

Specialty section:

This article was submitted to
Livestock Genomics,
a section of the journal
Frontiers in Genetics

Received: 13 December 2017

Accepted: 23 March 2018

Published: 10 April 2018

Citation:

Xu S-S, Gao L, Xie X-L, Ren Y-L,
Shen Z-Q, Wang F, Shen M,
Eyþórsdóttir E, Hallsson JH,
Kiseleva T, Kantanen J and Li M-H
(2018) Genome-Wide Association
Analyses Highlight the Potential
for Different Genetic Mechanisms
for Litter Size Among Sheep Breeds.
Front. Genet. 9:118.
doi: 10.3389/fgene.2018.00118

¹ CAS Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences (CAS), Beijing, China, ² College of Life Sciences, University of Chinese Academy of Sciences, Beijing, China, ³ Institute of Animal Husbandry and Veterinary Medicine, Xinjiang Academy of Agricultural and Reclamation Science, Shihezi, China, ⁴ State Key Laboratory of Sheep Genetic Improvement and Healthy Breeding, Xinjiang Academy of Agricultural and Reclamation Science, Shihezi, China, ⁵ Shandong Binzhou Academy of Animal Science and Veterinary Medicine Academy, Binzhou, China, ⁶ Institute of Sheep and Goat Science, Nanjing Agricultural University, Nanjing, China, ⁷ Faculty of Natural Resources and Environmental Sciences, Agricultural University of Iceland, Borgarnes, Iceland, ⁸ All-Russian Research Institute of Genetics and Farm Animal Breeding, Russian Academy of Sciences, Moscow, Russia, ⁹ Production Systems, Natural Resources Institute Finland, Jokioinen, Finland

Reproduction is an important trait in sheep breeding as well as in other livestock. However, despite its importance the genetic mechanisms of litter size in domestic sheep (*Ovis aries*) are still poorly understood. To explore genetic mechanisms underlying the variation in litter size, we conducted multiple independent genome-wide association studies in five sheep breeds of high prolificacy (Wadi, Hu, Icelandic, Finnsheep, and Romanov) and one low prolificacy (Texel) using the Ovine Infinium HD BeadChip, respectively. We identified different sets of candidate genes associated with litter size in different breeds: *BMPR1B*, *FBN1*, and *MMP2* in Wadi; *GRIA2*, *SMAD1*, and *CTNNA1* in Hu; *NCOA1* in Icelandic; *INHBB*, *NF1*, *FLT1*, *PTGS2*, and *PLCB3* in Finnsheep; *ESR2* in Romanov and *ESR1*, *GHR*, *ETS1*, *MMP15*, *FLI1*, and *SPP1* in Texel. Further annotation of genes and bioinformatics analyses revealed that different biological pathways could be involved in the variation in litter size of females: hormone secretion (FSH and LH) in Wadi and Hu, placenta and embryonic lethality in Icelandic, folliculogenesis and LH signaling in Finnsheep, ovulation and preovulatory follicle maturation in Romanov, and estrogen and follicular growth in Texel. Taken together, our results provide new insights into the genetic mechanisms underlying the prolificacy trait in sheep and other mammals, suggesting targets for selection where the aim is to increase prolificacy in breeding projects.

Keywords: sheep, prolificacy, genome-wide association study, biological pathways, regulation

INTRODUCTION

Reproduction is one of the most important traits in livestock production particularly for females. Selection for higher prolificacy in domestic sheep (*Ovis aries*) has led to variable litter size (LS) within and among breeds. For example, individual litter size of 1 to 8 has been recorded in the Hu sheep and Finnsheep (Yue, 1996; Davis et al., 2006a).

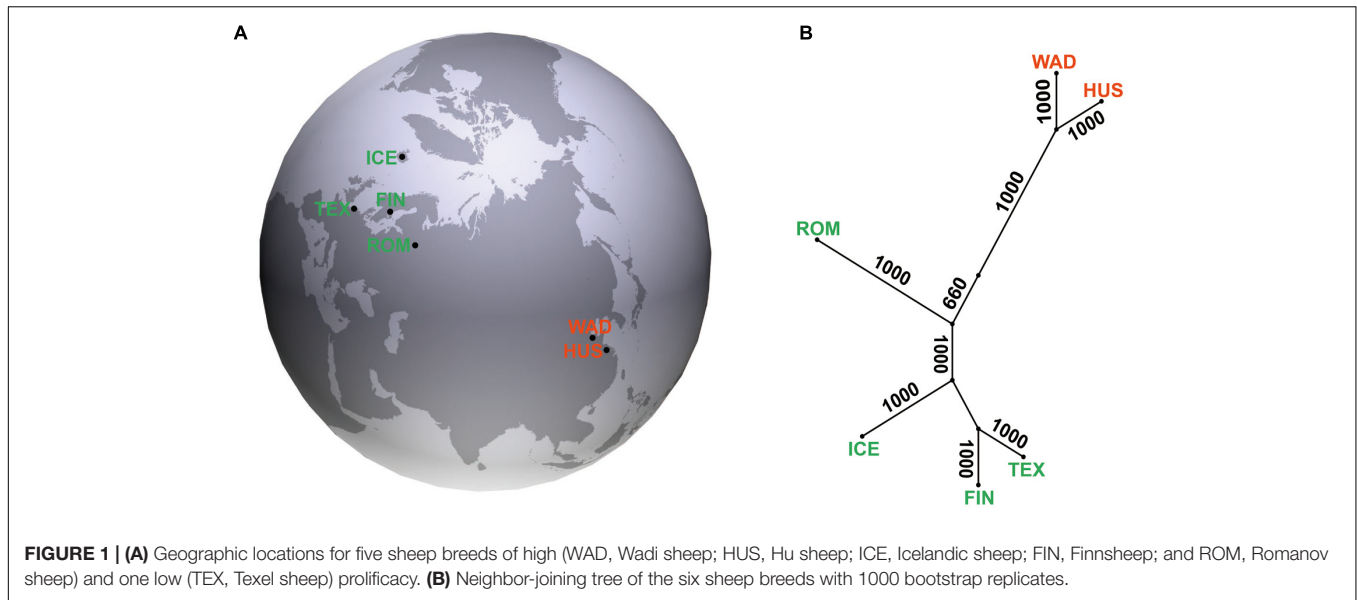
Previous studies reported that the exceptional prolificacy of the Booroola Merino was attributed to a single major gene, while a number of mutations of a major effect on litter size have been identified in other sheep breeds (Table 1; see also Xu and Li, 2017). Vage et al. (2013) detected a mutation $FecG^F$ in gene *GDF9* strongly associated with litter size in Norwegian White Sheep and Finnish Landrace (Finnsheep) using a genome-wide association analysis. Demars et al. (2013) reported the mutations $FecX^{Gr}$ in Grivette sheep and $FecX^O$ in Olkuska sheep associated with the highly prolific phenotype by a genome-wide association analysis. Cao et al. (2016) found that nine candidate genes including the well-known *FecB* mutation played important roles in the variable litter size in Hu and Small-tailed Han sheep through methylated DNA-immunoprecipitation sequencing data. Miao et al. (2016) identified a set of differentially expressed genes (e.g., *FecB*) between low- and high-prolificacy breeds (Dorset vs. Small-tailed Han sheep) through implementing integrated analysis of miRNAs and lncRNAs. Lassoued et al. (2017) found the mutation $FecX^{Bar}$ associated with the prolificacy in Tunisian Barbarine. Despite its great importance the genetic mechanisms of the high prolificacy

trait in domestic sheep are still poorly understood, partly due to shortage of studies conducted across multiple prolific sheep breeds. To date, numerous fecundity-associated mutations have been identified in different sheep breeds, but very few mutations have been consistently detected across the breeds. Despite the reproduction of ewes can be affected by the complex interactions of environmental conditions (i.e., climate, density, and food abundance) (Wilson et al., 2009), previous studies suggested that genetic factor could play important roles in the variable litter size of ewes.

In this study, we conducted multiple independent genome-wide association studies (GWAS) on litter size in the sheep breeds of high (Wadi, Hu, Icelandic, Finnsheep, and Romanov) and low (Texel) prolificacy with a litter size ranging from 1 to 6 from different geographic regions (Figure 1A) and genetic origins (Figure 1B) of the world, respectively. Wadi sheep is a high-prolificacy native breed from the Shandong Province of China (Peng et al., 2017). Hu sheep is famous for early sexual maturity and high fecundity, and are distributed in the Taihu Lake area of Eastern China (Yue, 1996). Icelandic and

TABLE 1 | Genetics variants associated with the fecundity in sheep.

Gene	Mutation	Name, allele symbol	Founder breeds	Reference
<i>BMP15</i>	V299D	Inverdale, $FecXI$	Romney, Inverdale	Galloway et al., 2000
	Q291Ter	Hanna, $FecXH$	Romney	Galloway et al., 2000
	S367I	Belclare, $FecXB$	Belclare	Hanrahan et al., 2004
	Q239R	Galway, $FecXG$	Belclare, Cambridge, Small-tailed Han	Hanrahan et al., 2004
	C321Y	Lacaune, $FecXL$	Lacaune	Bodin et al., 2007
	$\Delta P154S159$	Rasa Aragonesa, $FecXR$	Rasa Aragonesa	Martinez-Royo et al., 2008; Monteagudo et al., 2009
	T317I	Grivette, $FecXGr$	Grivette (France)	Demars et al., 2013
	N337H	Olkuska, $FecXO$	Olkuska (Poland)	Demars et al., 2013
	c.301G > T, c.310insC, c.302_304delCTA	Barbarine, $FecXBar$	Tunisian Barbarine	Lassoued et al., 2017
	Unknown	Woodlands, $FecXW$	Woodlands	Feary et al., 2007
<i>BMPR1B</i>	Q249R	Booroola, $FecBB$	Booroola Merino, Garole, Javanese, Small-tailed Han, Wadi, Hu	Mulsant et al., 2001; Souza et al., 2001; Wilson et al., 2001; Chu et al., 2011; Zhang et al., 2011; Cao et al., 2016
	S395F	High Fertility, $FecGH$	Belclare, Cambridge	Hanrahan et al., 2004
<i>GDF9</i>	S427R	Thoka, $FecGT$	Icelandic	Nicol et al., 2009
	F345C	Embrapa, $FecGE$	Santa Ines	Silva et al., 2011
	V371M	$FecGF$	Norwegian White Sheep, Finnsheep Landrace, Belclare	Vage et al., 2013; Mullen and Hanrahan, 2014
	R315C	Vacaria, $FecGV$	Brazilian sheep	de Souza et al., 2012
	R87H	$FecGI$	Baluchi	Moradband et al., 2011
<i>B4GALNT2</i>		Lacaune, $FecLL$	Lacaune	Drouilhet et al., 2013
Woodlands		Wood-land, $FecX2W$	Coopworth	Davis, 2005
<i>OLKUSKA</i>			Olkuska	Davis, 2004
<i>BELLE-ILE</i>			Belle-Ile	Davis, 2005
Unknown		$FecW$		Davis et al., 2006b



Finnsheep (Finnish Landrace) sheep are northern European high-fecundity breeds (Mullen and Hanrahan, 2014; Eiriksson and Sigurdsson, 2017). Romanov sheep from the Volga Valley shows outstanding reproduction qualities: early sexual maturity, out-of-season breeding and extraordinary prolificacy (Deniskova et al., 2017). The Texel sheep is a relatively low-prolificacy breed originally from the island of Texel in the Netherlands and excels in muscle growth and lean carcasses (Casas et al., 2004). Our results will be important for further genetic improvement of the trait and for better understanding the molecular basis of reproduction in sheep as well as other mammals.

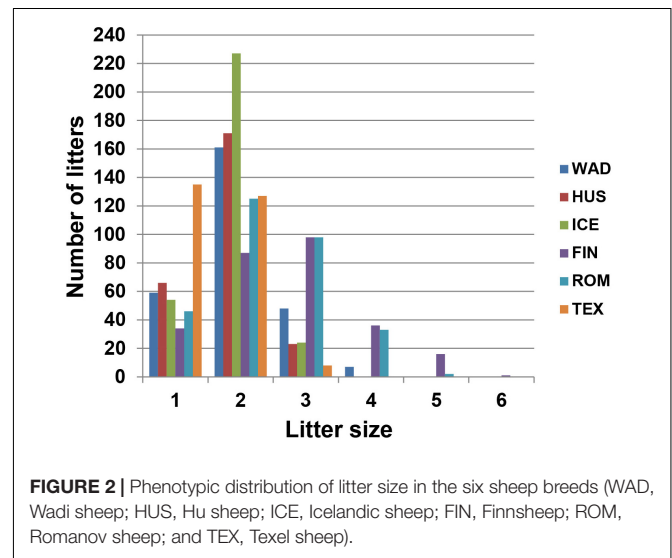
MATERIALS AND METHODS

Sample Collection and Phenotyping

A total of 522 ewes from five sheep breeds of high (Wadi, $n = 160$; Hu, $n = 117$; Icelandic, $n = 54$; Finnsheep, $n = 54$; and Romanov, $n = 78$) and one low (Texel, $n = 59$) prolificacy were collected from farms in China, Iceland, Finland, and Russia (Figure 1A). Animals included were as unrelated as possible based on analysis of pedigree records and farmers' knowledge. Data for the phenotype of litter size and the total number of litters collected from farm records are shown in Figure 2. The litter size ranged from 1 to 6 based on parity from 1 to 11 in six sheep breeds. Genomic DNA was extracted from the ear marginal tissues following a standard phenol/chloroform method and was diluted to 50 ng/μl for the SNP BeadChip genotyping (Köchler et al., 2005), except for the Icelandic samples which were isolated from whole-blood using MasterPure™ Complete DNA Purification Kit (Epicentre Biotech) following the manufacturers protocol.

Genotyping and Quality Control

All the samples were genotyped using the Ovine Infinium HD BeadChip according to the manufacturer's protocol. Genotypes of



a total of 606,006 SNPs were obtained (genotype and phenotype datasets¹). We implemented quality control of these SNPs using PLINK v1.07 software (Purcell et al., 2007). The SNPs or individuals were excluded if they met any of the criteria: (1) no chromosomal or physical location, (2) call rate < 0.95, (3) missing genotype frequency > 0.05, and/or (4) minor allele frequency (MAF) < 0.05. SNPs were excluded from the analysis if a p -value of Fisher's exact test for Hardy-Weinberg equilibrium less than 0.001.

Genetic Relationships and Population Structure

To investigate the genetic relationships and population structure among the six domestic sheep, we performed global F_{ST} ,

¹<https://www.animalgenome.org/repository/pub/CAAS2018.0302/>

neighbor-joining (NJ) tree and principle component analysis (PCA). The global F_{ST} value was calculated using GENEPOP v4.2 (Raymond and Rousset, 1995). The genetic distances between populations were calculated using an identity by state (IBS) similarity matrix (Kang et al., 2010). Then, the distances were used to construct a NJ tree with 1000 bootstraps using the package PHYLIP v.3.695 (Felsenstein, 1989). In addition, PCA was conducted using the SmartPCA program from the EIGENSOFT package version 4.2 (Patterson et al., 2006) based on the genotypes data.

Genome-Wide Association Analysis

To explore genetic structure within the breeds, multidimensional scaling (MDS) analysis was performed based on the independent SNPs using PLINK v1.07. Firstly, we implemented the option of 'indep-pairwise 50 5 0.05' in PLINK v1.07, which calculated pairwise linkage disequilibrium (LD) in a 50-SNP-window shifted at a pace of five SNPs. If the LD estimate was $r^2 > 0.05$, one of the pairs of SNPs was removed (Purcell et al., 2007). The independent SNPs retained by the LD criteria were then used in the MDS analysis, and the results were plotted using the GenABEL package in R v3.2.2 (Aulchenko et al., 2007).

We performed genome-wide association studies within five sheep breeds of high prolificacy (Wadi, Hu, Icelandic, Finnsheep, and Romanov) and one low prolificacy (Texel) using the case/control design. We ranked all individuals within the breeds according to their litter size from the highest to lowest. Then, we selected individuals from two tails for each breed as 'case' and 'control,' respectively. Based on the distribution of phenotypes, 114 samples ($LS \geq 2$) in Wadi, 66 samples ($LS \geq 2$) in Hu, 20 samples ($LS > 2$) in Icelandic, 37 samples ($LS \geq 2.5$) in Finnsheep, 40 samples ($LS \geq 2.5$) in Romanov and 28 samples ($LS \geq 1.6$) in Texel sheep were selected as 'cases,' while 28 samples ($LS = 1$) in Wadi, 15 samples ($LS = 1$) in Hu, 15 samples ($LS \leq 1.75$) in Icelandic, 9 samples ($LS \leq 2$) in Finnsheep, 26 samples ($LS \leq 2$) in Romanov and 14 samples ($LS \leq 1.33$) in Texel sheep were selected as 'controls.' In the GWAS, we used the function of "qtscore" in the GenABEL package. Associated SNPs were identified at both the genome-wide and chromosome-wide significance levels ($p < 0.05$) after the Bonferroni correction (Bonferroni, 1936). To account for systematic biases caused by within-population substructure, the first and second dimensions from the MDS analyses were used as the covariates (Price et al., 2006). The correlation analysis between litter size and parity within breeds showed that there were significant effects between litter size and parity in four breeds (Wadi, Hu, Icelandic, and Texel), and the effect of parity 1 on litter size was less than that of parities 2 through 10 (Supplementary Table S1 and Supplementary Figure S1). However, the parity of individuals within breeds was different, and we mainly focused on the mean of litter size of individual (total litter size/parity) in per breed. Therefore, we excluded the effect of parity from the model. The Quantile-Quantile (Q-Q) plots were visualized by plotting the distribution of obtained vs. expected genome-wide p -values. For genotype effect of potential SNPs on litter size in each breed, differences between means were analyzed by the Student's t -test. The $p < 0.05$ was considered statistically significant. All

the results were presented as mean \pm standard error (SE). We implemented pairwise tests of linkage disequilibrium (LD) between the most significant SNPs and their flanking SNPs within approximately 1 Mb upstream and downstream using PLINK v1.07. Regional association plots were generated using the R package v3.2.2.

Bioinformatics Analysis

We annotated the genes associated with litter size in each breed using the *O. aries* assembly Oar_v.4.0². Further, we submitted the genes to the DAVID (database for annotation, visualization and integrated discovery) database³ for gene ontology (GO) enrichment and pathways analyses (Huang et al., 2009a,b). The p -value of 0.1 and at least two genes from the input gene list in the enriched category were considered for the enriched GO terms. Also, we investigated the protein-protein interaction network for the candidate genes using the STRING database version 10.5 (Szklarczyk et al., 2017). In addition, differential expressions of the candidate genes in various tissues were examined using the EMBL-EBI Expression Atlas database⁴ (Petryszak et al., 2016).

RESULTS

Population Relationship and Differentiation

Pairwise F_{ST} value varied from 0.023 to 0.104 among the populations with the least genetic differentiation observed between Wadi and Hu sheep breeds (Supplementary Table S2). The NJ tree showed that these breeds were clustered into two major groups according to their Chinese and European origins (Figure 1B). A similar geographic pattern was seen in the PCA analyses with the grouping of Wadi and Hu sheep separated from the other four European breeds (Supplementary Figure S2).

Genome-Wide Association Analysis

After the quality control, 508,444 SNPs and 114 individuals (91 cases vs. 23 controls) in Wadi, 506,031 SNPs and 80 individuals (66 cases vs. 14 controls) in Hu, 443,125 SNPs and 23 individuals (8 cases vs. 15 controls) in Icelandic, 492,165 SNPs and 37 individuals (28 cases vs. 9 controls) in Finnsheep, 465,794 SNPs and 38 individuals (29 cases vs. 9 controls) in Romanov, 475,955 SNPs and 39 individuals (28 cases vs. 11 controls) in Texel sheep were retained in the working dataset for the GWAS. We did find several animals outlying the clusters of cases, which might cause biases in the association analyses (Supplementary Figure S3). We have repeated the association analyses without these animals, and found the results are very similar. Thus, we did not exclude these animals in the association analyses due to the small sample size for the breeds. The resulting genomic inflation factors were equal to 1.07 in Wadi, 1.14 in Hu, 1.12 in Icelandic, 1.14 in Finnsheep, 1.10 in Romanov, and 1.05 in

²<http://www.ncbi.nlm.nih.gov/genome?term=ovis%20aries>

³<https://david.ncifcrf.gov/>

⁴<https://www.ebi.ac.uk/gxa/home/>

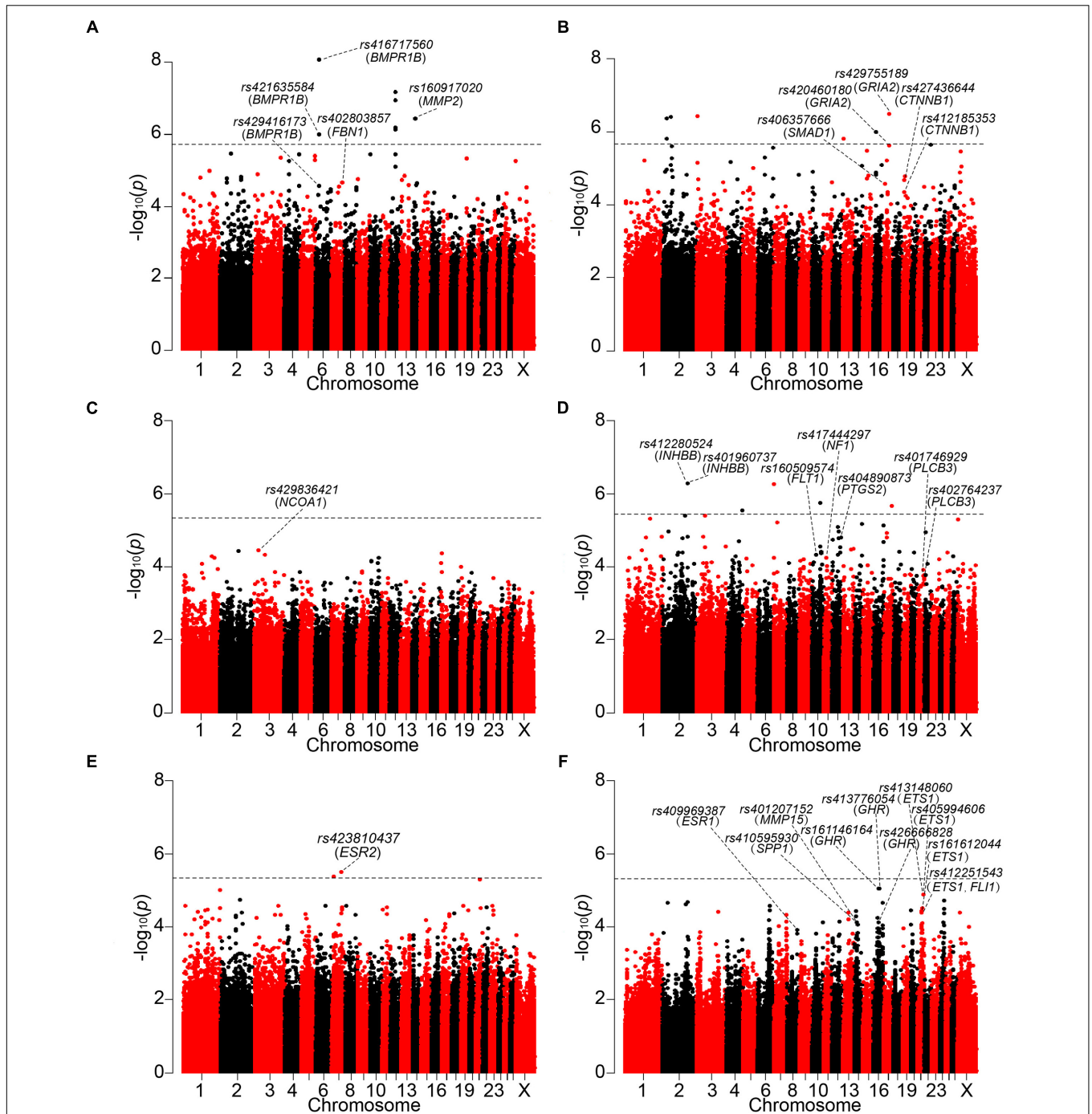
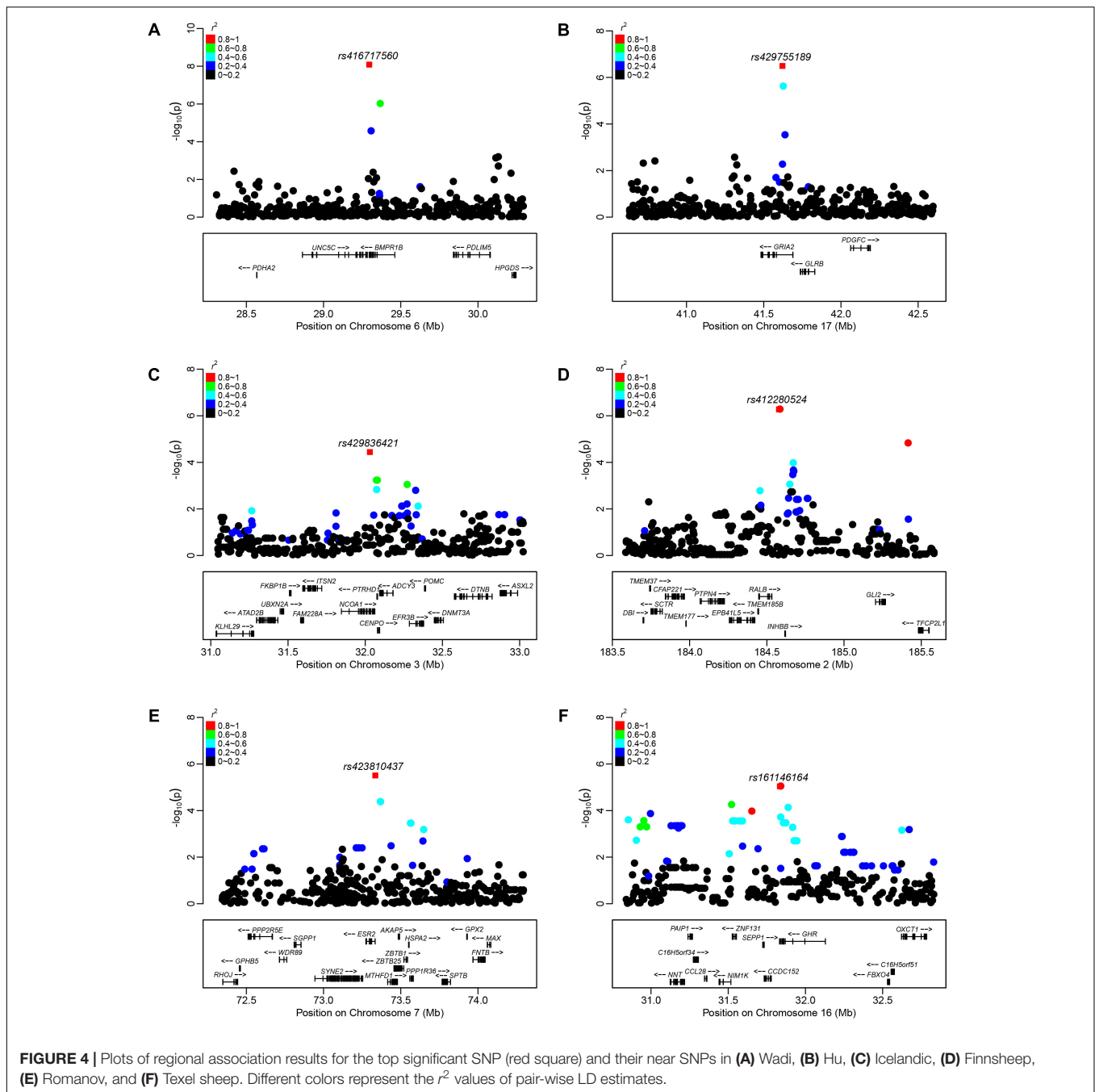


FIGURE 3 | Manhattan plots of GWAS are shown on (A) Wadi, (B) Hu, (C) Icelandic, (D) Finnsheep, (E) Romanov and (F) Texel sheep. The 5% genome-wide significant threshold value is indicated by a dotted line. The significant SNPs surrounding the genes previously reported to be associated with reproduction are annotated at the chromosome-wise and genome-wide 5% significance after the Bonferroni correction.

Texel sheep, suggesting well-controlled population stratifications (Supplementary Figure S4).

In Wadi sheep, we detected 59 and 8 SNPs at the chromosome-wise and genome-wide ($p < 1.92 \times 10^{-6}$) 5% significance after the Bonferroni correction, respectively (Figure 3A and Supplementary Tables S3, S4). We observed a high level of LD

between the top significant SNP *rs416717560* and *rs421635584* located in gene *BMP1B* (Figure 4A). For the SNP *rs416717560*, average litter size of individuals with the G/G genotype ($n = 115$, $LS = 2.05 \pm 0.06$) was significantly ($p < 0.01$) higher than that of the ewes with the A/G ($n = 15$, $LS = 1.47 \pm 0.16$) genotype (Figure 5A). Also, we found three additional significant

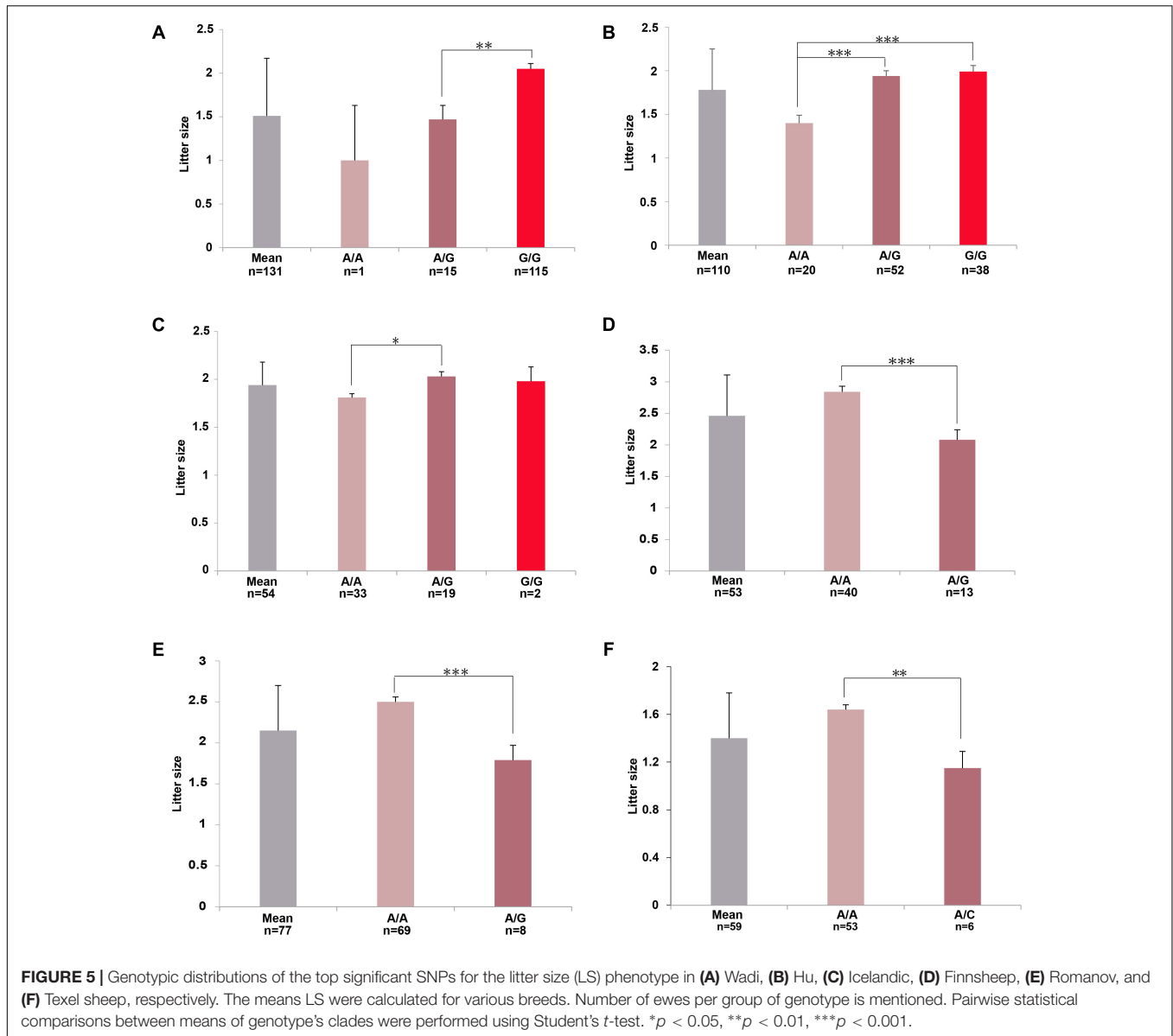


SNPs (*rs429416173*, *rs402803857*, and *rs160917020*) neighboring genes *BMPR1B*, *FBN1*, and *MMP2* (Table 2 and Supplementary Table S3).

In Hu sheep, we identified 98 and 9 SNPs at the chromosome-wise and genome-wide ($p < 2.18 \times 10^{-6}$) 5% significance after Bonferroni correction (Figure 3B and Supplementary Tables S3, S4). The regional plot showed that the top significant SNPs *rs429755189* and *rs420460180* on chromosome 17 were in an LD block that contained gene *GRIA2* (Figure 4B). For the *rs429755189*, average litter size of individuals with the genotypes G/G ($n = 38$, $LS = 1.99 \pm 0.07$) and A/G ($n = 52$, $LS = 1.94 \pm 0.06$)

were significantly ($p < 0.001$) higher than that of ewes with the genotype A/A ($n = 20$, $LS = 1.40 \pm 0.09$) in the present population (Figure 5B). Among these significant SNPs, 3 (*rs406357666*, *rs427436644* and *rs412185353*) are located within the genes *SMAD1* and *CTNNB1* (Table 2 and Supplementary Table S3).

In Icelandic sheep, we found 22 SNPs at the chromosome-wise 5% significance after the Bonferroni correction (Figure 3C and Supplementary Tables S3, S4). The top significant SNP *rs429836421* on chromosome 3 was located within gene *NCOA1* (Figure 4C). For *rs429836421*, average litter size of individuals with the A/G genotype ($n = 19$, $LS = 2.03 \pm 0.05$) is significantly



(*p* < 0.05) higher than that of the ewes with the genotype A/A (*n* = 33, LS = 1.81 ± 0.04) (Figure 5C).

In Finnsheep, we detected 102 and 6 SNPs at the chromosome-wise and genome-wide (*p* < 3.64 × 10⁻⁶) 5% significance after the Bonferroni correction, respectively (Figure 3D and Supplementary Tables S3, S4). The regional plot revealed strong LD between the top significant SNP *rs412280524* and its neighboring SNPs *rs401960737* and *rs407751830* harbored gene *INHBB* (Figure 4D). For the SNP *rs412280524*, litter size of ewes with the genotype A/A (*n* = 40, LS = 2.84 ± 0.09) is significantly (*p* < 0.001) higher than that of the ewes with the genotype A/G (*n* = 13, LS = 2.08 ± 0.16) (Figure 5D). Also, five additional significant SNPs (*rs160509574*, *rs417444297*, *rs404890873*, *rs401746929*, and *rs402764237*) were found to be located near to genes *FLT1*, *NF1*, *PTGS2*, and *PLCB3* (Table 2 and Supplementary Table S3).

In Romanov sheep, we identified 77 and 2 SNPs at the chromosome-wise and genome-wide (*p* < 4.56 × 10⁻⁶) 5% significance after the Bonferroni correction (Figure 3E and Supplementary Tables S3, S4). The top significant SNP *rs423810437* on chromosome 7 was in the gene *ESR2* (Figure 4E). For *rs423810437*, litter size of ewes with the genotype A/A (*n* = 69, LS = 2.50 ± 0.06) is significantly (*p* < 0.001) higher than that of the ewes with the genotype A/G (*n* = 8, LS = 1.79 ± 0.18) (Figure 5E).

In Texel sheep, we observed 133 SNPs at the chromosome-wise 5% significance after the Bonferroni correction (Figure 3F and Supplementary Tables S3, S4). The regional plot showed that the top significant SNPs *rs161146164* and *rs413776054* on chromosome 16 were in a strong LD region containing one functional gene *GHR* (Figure 4F). For *rs161146164*, litter size of ewes with the genotype A/A (*n* = 53, LS = 1.64 ± 0.05)

TABLE 2 | Genome-wide and chromosome-wise significant SNPs and associated genes.

Population	SNP	Chr	Position (bp)	MAF	<i>p</i> -unadjusted	<i>p</i> -adjusted	Genes	Location
Wadi	<i>rs416717560*</i>	6	29295803	0.07	3.65E-08	8.19E-09	<i>BMPR1B</i> ¹	3'UTR
	<i>rs421635584*</i>	6	29361782	0.05	4.36E-06	9.78E-07	<i>BMPR1B</i> ¹	Intron
	<i>rs429416173</i>	6	29302788	0.2	7.55E-05	2.75E-05	<i>BMPR1B</i> ¹	CDS
	<i>rs402803857</i>	7	58598895	0.1	4.96E-05	2.93E-05	<i>FBN1</i> ¹	Intron
	<i>rs160917020*</i>	14	23133427	0.19	1.10E-06	3.71E-07	<i>MMP2</i>	Downstream
Hu	<i>rs429755189*</i>	17	41621298	0.43	1.94E-06	3.21E-07	<i>GRIA2</i> ¹	Intron
	<i>rs420460180</i>	17	41621269	0.29	8.50E-06	2.43E-06	<i>GRIA2</i> ¹	Intron
	<i>rs406357666</i>	17	12487861	0.19	1.40E-05	2.66E-05	<i>SMAD1</i> ¹	Intron
	<i>rs427436644</i>	19	13639996	0.32	7.69E-05	2.14E-05	<i>CTNNB1</i>	Downstream
	<i>rs412185353</i>	19	13641870	0.33	1.51E-04	4.49E-05	<i>CTNNB1</i>	Downstream
Icelandic	<i>rs429836421</i>	3	32030054	0.16	4.55E-05	3.63E-05	<i>NCOA1</i> ¹	Intron
Finnsheep	<i>rs412280524*</i>	2	184578329	0.09	2.62E-05	5.32E-07	<i>INHBB</i>	Downstream
	<i>rs401960737*</i>	2	184579671	0.09	2.62E-05	5.32E-07	<i>INHBB</i>	Downstream
	<i>rs160509574</i>	10	31933001	0.27	1.50E-05	4.71E-05	<i>FLT1</i> ¹	Intron
	<i>rs417444297</i>	11	18552961	0.11	4.20E-05	5.65E-05	<i>NF1</i>	Downstream
	<i>rs404890873</i>	12	65662842	0.05	1.87E-04	1.59E-05	<i>PTGS2</i>	Upstream
	<i>rs401746929</i>	21	41915064	0.08	1.85E-03	1.75E-04	<i>PLCB3</i>	Upstream
	<i>rs402764237</i>	21	41919836	0.08	1.85E-03	1.75E-04	<i>PLCB3</i>	Upstream
	Romanov	<i>rs423810437*</i>	7	73335157	0.07	1.65E-05	3.12E-06	<i>ESR2</i> ¹
Texel	<i>rs409969387</i>	8	75353388	0.08	1.11E-03	1.21E-04	<i>ESR1</i>	Intron
	<i>rs410595930</i>	14	23645021	0.06	1.33E-04	1.46E-04	<i>SPP1</i> ¹	Intron
	<i>rs401207152</i>	14	25147418	0.06	1.33E-04	1.46E-04	<i>MMP15</i>	Downstream
	<i>rs161146164</i>	16	31834495	0.06	1.33E-04	9.11E-06	<i>GHR</i> ¹	CDS
	<i>rs413776054</i>	16	31834942	0.06	1.33E-04	9.11E-06	<i>GHR</i>	CDS
	<i>rs426666828</i>	16	31882869	0.18	1.88E-04	7.54E-05	<i>GHR</i> ¹	Intron
	<i>rs413148060</i>	21	30950537	0.15	1.02E-04	4.17E-05	<i>ETS1</i>	Upstream
	<i>rs405994606</i>	21	31001548	0.15	1.02E-04	4.17E-05	<i>ETS1</i> ¹	Intron
	<i>rs161612044</i>	21	31009743	0.14	5.41E-04	1.01E-04	<i>ETS1</i> ¹	Intron
	<i>rs412251543</i>	21	31178275	0.1	4.01E-03	1.46E-04	<i>ETS1/FLI1</i>	Upstream/Downstream

For genes the best SNP of which is located outside of upstream/downstream 150 kb region. Chr., chromosome; MAF, Minor Allele Frequency. The *p*-unadjusted corresponds to exact *p* for the Fisher's test. The *p*-adjusted corresponds to the corrected significance of GWAS after principle component adjustment. The SNPs with symbol (*) denote that bonferroni-corrected genome-wide significant SNPs. The genes with symbol (1) denote that the SNPs are intragenic, otherwise they are the nearest genes upstream and downstream of the tested SNPs.

is significantly (*p* < 0.01) higher than that of the ewes with the genotype A/C (*n* = 6, LS = 1.15 ± 0.14) (Figure 5F). The two mutations (*rs161146164*, Asn > His; *rs413776054*, Pro > Ser) cause the amino acid change in coding region of the *GHR* gene. In addition, we found eight additional significant SNPs (*rs426666828*, *rs409969387*, *rs410595930*, *rs401207152*, *rs413148060*, *rs405994606*, *rs161612044*, and *rs412251543*) surrounding genes *ESR1*, *ETS1*, *FLI1*, *SPP1*, and *MMP15* (Table 2 and Supplementary Table S3).

In addition to the source breed where the target SNPs have been detected, we further assessed genotype effect of the most significant SNPs on litter size in the other five sheep breeds. In general, genotypes of the target SNPs did not show significant association with increased litter size in the breeds other than the source breed (Supplementary Table S7). Nevertheless, we observed some exceptions. For example, the genotype A/G of *rs429836421*, which was identified in Icelandic sheep, showed significant associations with increased litter size in both Icelandic and Hu sheep breeds. However, a lack of homozygotes for the SNPs such as the genotype

G/G for *rs412280524* in Finnsheep, G/G for *rs423810437* in Romanov and C/C for *rs161146164* in Texel sheep could be because of low frequency of the mutations and small sample size.

Bioinformatics Analysis

We found significantly (*p* < 0.1) enriched GO terms associated with reproduction for the candidate genes. The GO clusters were primarily enriched in the categories of ovarian and oocyte development (*PTGS2*, *BMPR1B*, *INHBB*, *CTNNB1*, *MMP2*, *MMP15*, *FBN1*, *GHR*, and *SPP1*), phospholipase C activity (*FLT1* and *ESR1*), SMAD protein (*INHBB* and *SMAD1*) and BMP signaling (*SMAD1* and *BMPR1B*) and positive regulation of transcription (*NCOA1*, *FLI1*, *ESR1*, *ESR2*, *CTNNB1*, *ETS1*, and *BMPR1B*), all of which are involved in the folliculogenesis, follicle growth and granulosa cell proliferation (Figure 6 and Supplementary Table S5). Another relevant GO category was hindbrain development (*SMAD1* and *CTNNB1*), which participated in regulating ovulation (Baird et al., 2006). In addition, we detected 11 genes (i.e., *PLCB3*, *ESR1*, *ESR2*,

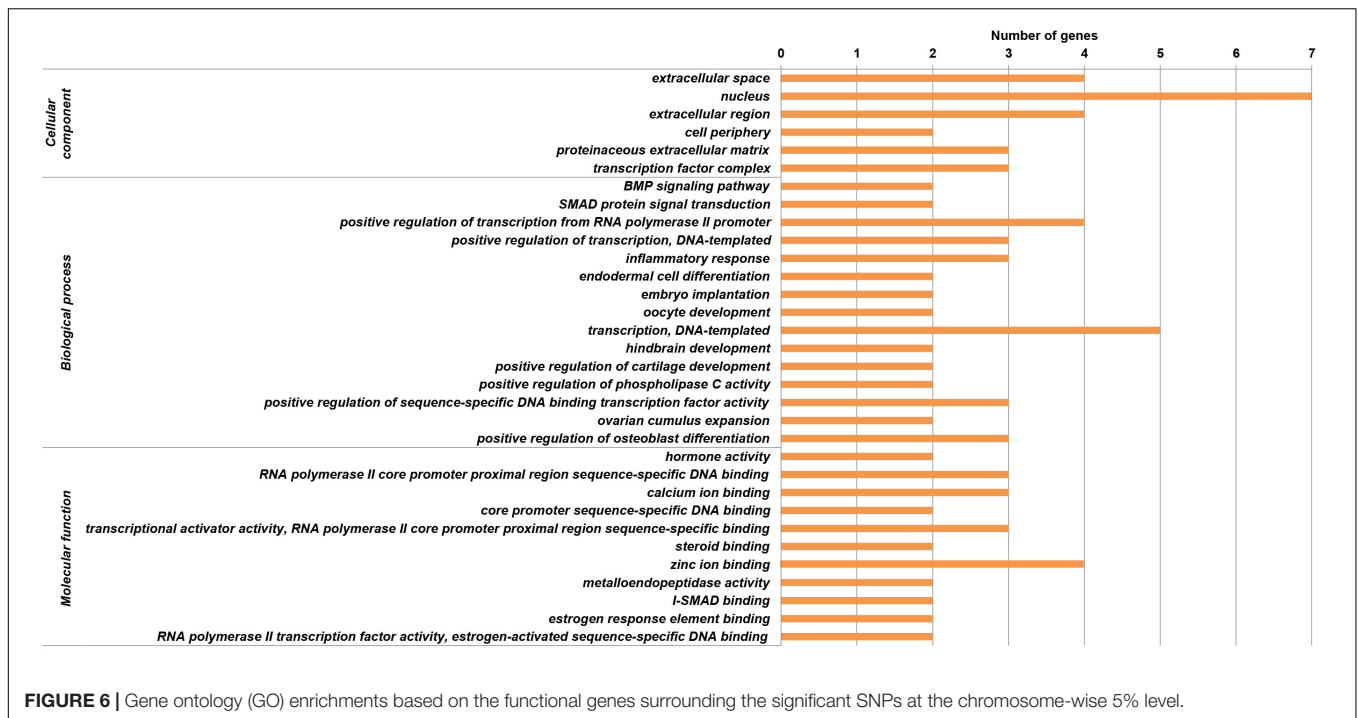


FIGURE 6 | Gene ontology (GO) enrichments based on the functional genes surrounding the significant SNPs at the chromosome-wise 5% level.

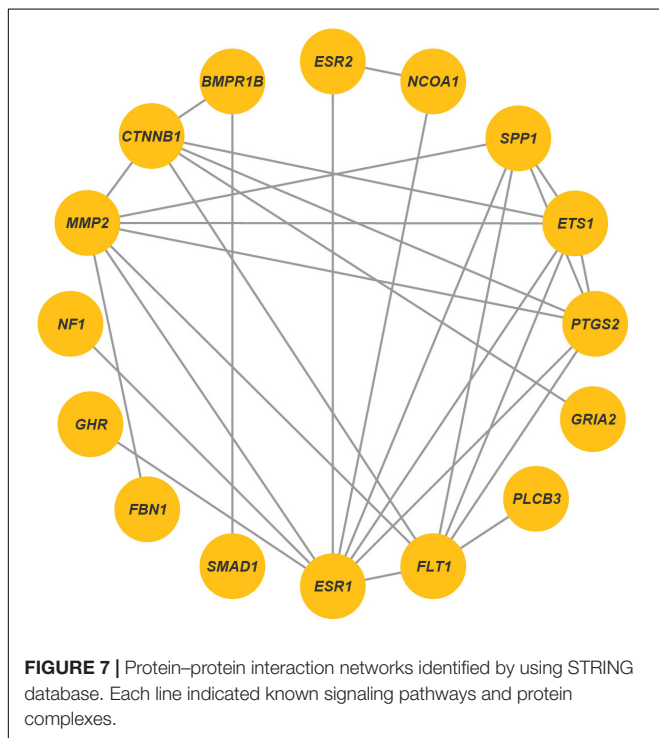


FIGURE 7 | Protein-protein interaction networks identified by using STRING database. Each line indicated known signaling pathways and protein complexes.

in different sheep breeds. For example, I-SMAD binding were enriched in Hu sheep, and chromatin binding were enriched in Texel sheep (**Supplementary Table S6**). In the gene network analysis, we observed that 16 genes (i.e., *BMPR1B*, *FBN1*, *MMP2*, *SMAD1*, *CTNNB1*, *GRIA2*, *NCOA1*, *FLT1*, *NF1*, *PTGS2*, *PLCB3*, *ESR2*, *ESR1*, *ETS1*, *SPP1*, and *GHR*) showed protein-protein interactions in the network (**Figure 7**). Expression data further showed that the genes *BMPR1B*, *FBN1*, *MMP2*, *GRIA2*, *SMAD1*, *CTNNB1*, *NCOA1*, *NF1*, *FLT1*, *PTGS2*, *PLCB3*, *ESR2*, *ESR1*, *GHR*, *ETS1*, *MMP15*, *FLII*, and *SPP1* were either highly or moderately expressed in reproduction-related tissues such as ovary, uterine cervix, placenta, corpus luteum, cerebellum, pituitary gland or uterus in sheep (**Figure 8**). Also, gene *INHBB* showed a high expression in ovary and uterus of *Mus musculus*⁵.

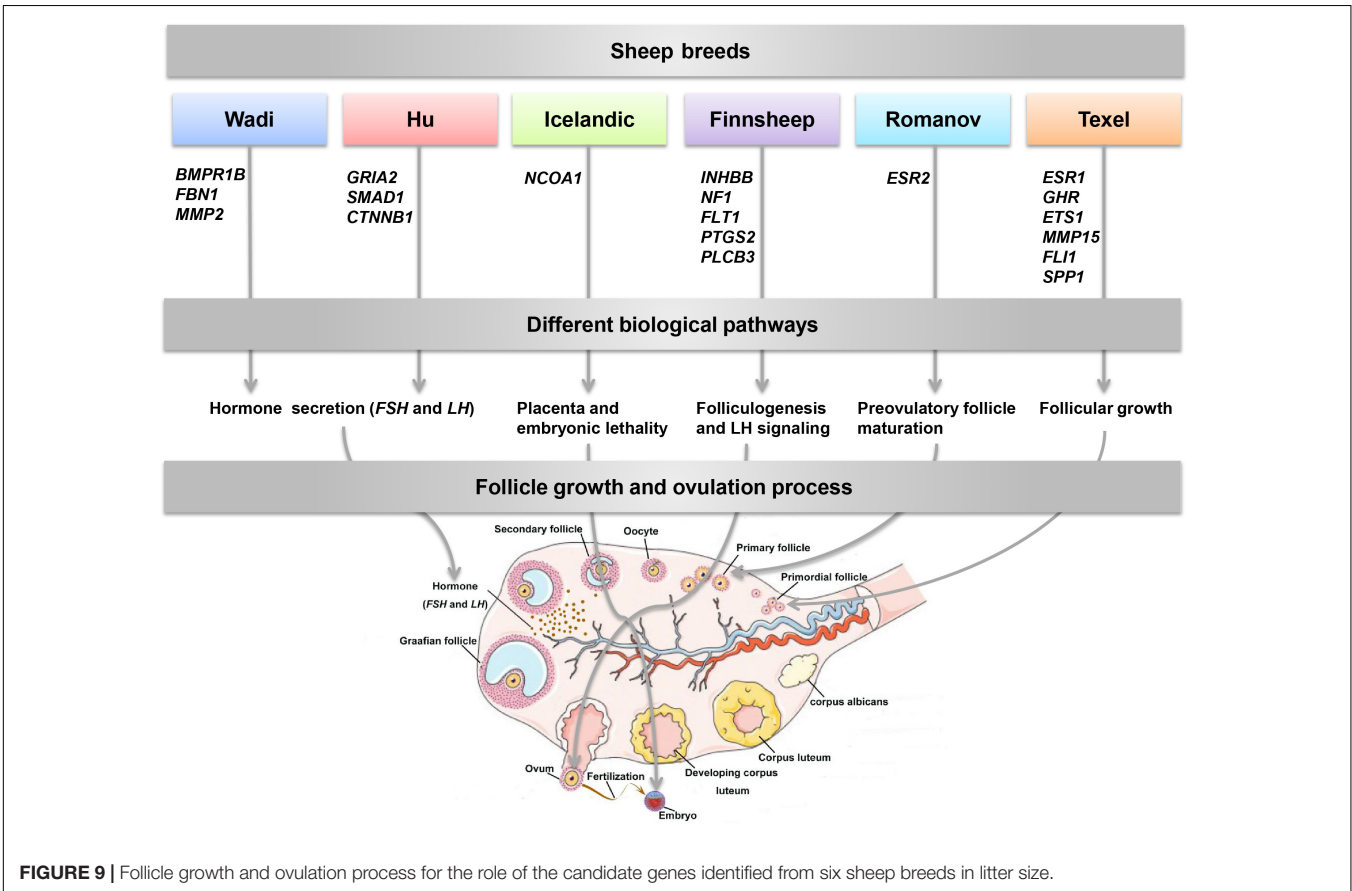
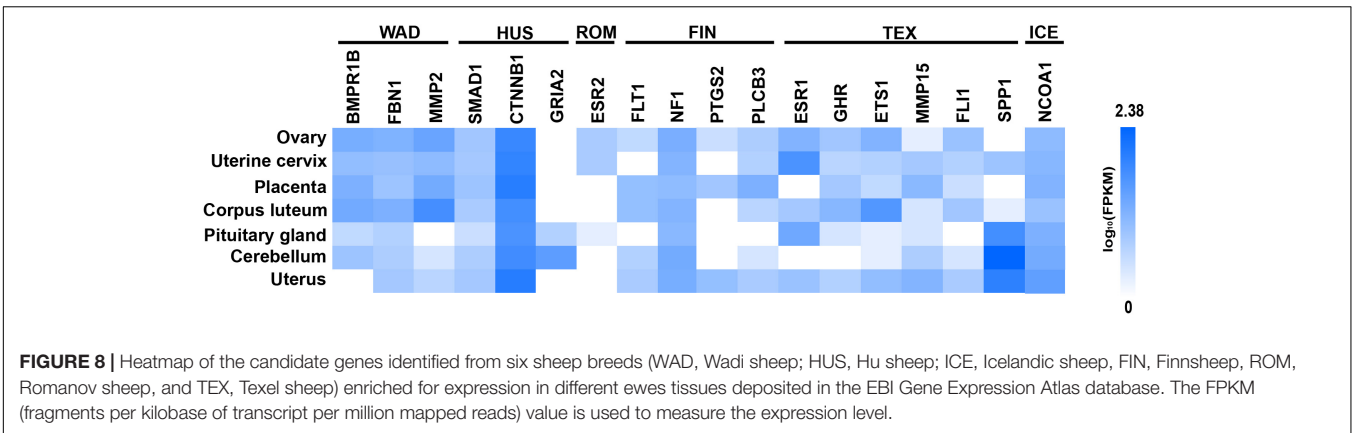
DISCUSSION

In this study, we conducted multiple independent GWAS in different sheep breeds to investigate the genetic mechanisms underlying the litter size in sheep. Coupled with population relationship and bioinformatics analyses, the GWAS identified different genes associated with the litter size in different breeds and revealed their differentially genetic regulation mechanisms associated with follicle growth and ovulation in the reproduction of ewes.

The diverse biological pathways identified from the novel genes annotation play an important role in follicle growth and ovulation of females in different sheep breeds (**Figure 9**). The three genes identified in Wadi sheep, *BMPR1B*, *FBN1*, and

MMP2, *NCOA1*, *CTNNB1*, *INHBB*, *SMAD1*, *BMPR1B*, *PTGS2*, and *GRIA2*) involved in estrogen, thyroid hormone, TGF-beta, retrograde endocannabinoid and hippo signaling pathways, and these pathways played important roles in regulating follicle growth and ovulation in livestock (**Supplementary Table S5**). However, we observed different GO terms for the candidate genes

⁵<https://www.ebi.ac.uk/gxa/home/>



MMP2, all play a crucial role in regulating hormone secretion (Mulsant et al., 2001; Basini et al., 2011; Zhang et al., 2011; Zhai et al., 2013). For example, *BMPR1B* gene can lead to an increased density of the follicle-stimulating hormone (FSH) and luteinizing hormone (LH) receptors with a concurrent reduction in apoptosis to increase the ovulation rate of ewes (Regan et al., 2015; Hu et al., 2016). As the main component of microfibrils in the extracellular matrix, the gene *FBN1* regulates cumulus cell apoptosis by reducing the expression level of *BMP15* involved in estrogen signaling in porcine ovaries (Zhai et al., 2013). The *MMP2* gene plays a key role in ovulation and follicle atresia

by regulating FSH and insulin like growth factor 1 (*IGF1*) (Knapp and Sun, 2017). In Hu sheep, the three genes *GRIA2*, *SMAD1*, and *CTNNB1* are related to estrogen response element (Chang et al., 2013; Kumar et al., 2016; Vastagh et al., 2016). For example, the gene *GRIA2* has been shown to participate in the glutamatergic pathway that regulates gonadotropin-releasing hormone (GnRH), a known prerequisite of the subsequent hormonal cascade inducing the ovulation in mice (Vastagh et al., 2016). The gene *SMAD1* encodes an intracellular BMP signaling molecule, which is involved in mediating ovulation rate of ewes (Xu et al., 2010). The *CTNNB1* gene enhances FSH and

LH actions in follicles by stimulating WNT/CTNNB1 pathway and G protein-coupled gonadotropin receptors in female (Fan et al., 2010). In Icelandic sheep, the gene *NCOA1* can alter the expression of multiple key genes *PBP*, *AIB3*, and *FGFR2*, which are important for aberrant labyrinth morphogenesis of the placenta and embryonic lethality (Chen et al., 2010; Huang et al., 2011). In Finnsheep, the five candidate genes *INHBB*, *NFI*, *FLT1*, *PTGS2*, and *PLCB3* played important roles in the development of folliculogenesis and LH signaling (Ding et al., 2006; Tal et al., 2014; De Cesaro et al., 2015; Ben Sassi et al., 2016; Cadoret et al., 2017). For example, the *INHBB* gene encodes an inhibitor of apoptosis, which regulates porcine ovarian follicular atresia (Terenina et al., 2017). The coding region of gene *NFI* presents non-CpG methylation in the murine oocyte, which plays a critical role in mammalian development (Haines et al., 2001). The *FLT1* gene has an important role in the activity of vascular endothelial growth factor that linked to folliculogenesis (Celik-Ozenci et al., 2003). The *PTGS2* gene plays a critical role in the ovulation by stimulating LH signaling in zebrafish (Tang et al., 2017). The *PLCB3* gene is highly expressed in bovine cells of the ovulatory-sized follicles, with the role of activating LH/LHR signaling (Castilho et al., 2014). In Romanov sheep, the gene *ESR2* activates ovulation and regulates preovulatory follicle maturation through regulating estrogen response element (Lalotiotis et al., 2017; Rumi et al., 2017). In Texel sheep, the six candidate genes *ESR1*, *GHR*, *ETS1*, *MMP15*, *FLII*, and *SPP1* are relevant to estrogen and follicular growth (Putnova et al., 2001; Bachelot et al., 2002; Munoz et al., 2007; Xiao et al., 2009; Hatzirodos et al., 2015; Ogiwara and Takahashi, 2017). As a key gene affecting estrogen biosynthesis, *ESR1* gene functions similarly to *ESR2*, and is critical for follicular growth and successful ovulation in ewes (Foroughinia et al., 2017). The *GHR* gene plays a role in follicular growth through stimulating *IGF1* in mice (Bachelot et al., 2002). The *ETS1* gene was linked to the regulator of protein signaling protein-2 (*RGS2*) involved in the ovulation in bovine (Sayasith et al., 2014). As a proteolytic enzyme gene, the *MMP15* gene has been shown to mediate LH and its receptor in the preovulatory follicles of teleost medaka (Ogiwara and Takahashi, 2017). The *FLII* gene encodes a critical transcription factor, which regulates gene *ETS1* (Vo et al., 2017). The *SPP1* gene accounts for establishing and maintaining cellular interactions between steroidogenic and non-steroidogenic cells during the development of corpus luteum (Poole et al., 2013). In addition, the GO categories as well as protein-protein network and expression analysis showed that these genes played an essential role in follicle growth and ovulation of ewes. However, further expression analyses of these genes in each breed are necessary in future study. Taken together, the apparent difference for the litter size among the breeds might be explained by diverse regulation mechanisms.

Also, we calculated genetic differentiation among populations using the global F_{ST} , PCA, and NJ tree methods to obtain a refined picture of population genetic relationships. The result showed that the genetic groups were consistent with the geographic origins of the breeds. The different genetic mechanisms associated with physiological processes for the

litter size among sheep breeds could be related to the various environments in different geographic regions.

We noticed that previous studies had identified several genes of major effect such as *BMPRI1B*, *BMP15*, and *GDF9* for the prolificacy in ewes (Table 1). Different from early investigations, we detected a set of novel genes for the litter size in ewes. The main reason could be that most of early studies are based on genome-wide selection tests between prolific and non-prolific breeds using a lower density of SNPs. Instead, here we implemented GWAS within specific sheep breeds of high or low prolificacy using a high density SNP BeadChip array, which should lead to more reliable associations. In addition, the difference in threshold value used to define the 'case' and 'control' groups for each breed was also another potentially influential factor. When we implemented the GWAS using a two-step approach via the general linear model and genome-wide efficient mixed-model analysis (GEMMA), we did not find interesting candidate genes associated with reproduction across the six breeds (see **Supplementary Material** for further details). The fact that no candidate genes associated with reproduction were detected could be due to that the power to detect such associations will be weak when treating the trait of interest as quantitative given the small sample size. Also, these populations could have been subjected to selection on litter size through environmental variables such as climate and diet. However, we did not obtain data for local environmental variables in our data. Thus, environmental variables as well as the age of reproduction for the ewes were not taken into account in the model of the GWAS, which would be essential for future study.

CONCLUSION

We revealed a set of novel functional genes for the litter size in different sheep breeds across the world. Our results suggested differentially genetic regulation mechanisms for the functional genes in the reproduction of sheep. The significant SNPs and genes identified here are useful for future molecular-based breeding for a higher fertility. Also, our results provide important insights into the regulation of reproduction in sheep and other mammals.

AUTHOR CONTRIBUTIONS

M-HL conceived and designed the project. FW, Z-QS, Y-LR, MS, EE, JH, JK, and TK collected the samples. X-LX extracted the DNA. JK provided help in Beadchip genotyping. S-SX and LG analyzed the data. S-SX wrote the paper with contributions from M-HL. All authors reviewed and approved the final manuscript.

FUNDING

This work was supported by grants from the National Natural Science Foundation of China (Grant Nos. 91731309 and 31661143014), the Taishan Scholars Program of Shandong

Province (No. ts201511085), the National Transgenic Breeding Project of China (2014ZX0800952B), the Academy of Finland (Grant No. 250633), and the Climate Genomics for Farm Animal Adaptation (ClimGen) Project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2018.00118/full#supplementary-material>

FIGURE S1 | Parity effect for litter size in the six breeds. X-axis is labeled as the number of parity and Y-axis represents litter size. Pairwise statistical comparisons between means of litter size in parity's clades were performed using Student's *t*-test. **p* < 0.05; ***p* < 0.01, and ****p* < 0.001.

FIGURE S2 | Principle component plots for 522 ewes from the six sheep breeds (WAD: Wadi sheep, HUS: Hu sheep, ICE: Icelandic sheep, FIN: Finnish sheep, ROM: Romanov sheep, and TEX: Texel sheep), respectively.

FIGURE S3 | Multidimensional scaling (MDS) plots in (a) Wadi, (b) Hu, (c) Icelandic, (d) Finnish, (e) Romanov, and (f) Texel sheep. The red squares indicate

animals from the case group (highly prolific ewes), and the purple dots represent animals in the control group (normally prolific ewes).

FIGURE S4 | Q-Q (quantile–quantile) plots of GWAS in (a) Wadi, (b) Hu, (c) Icelandic, (d) Finnish, (e) Romanov, and (f) Texel sheep. Gray and black rings represent association statistics before and after correction for population stratification, respectively.

TABLE S1 | Parity effect for litter size and pairwise statistical comparisons between means of litter size in parity's clades in the six breeds.

TABLE S2 | Pairwise F_{ST} value among six breeds.

TABLE S3 | Bonferroni-corrected 5% chromosome-wise significance threshold in the six sheep breeds, respectively.

TABLE S4 | Bonferroni-corrected genome-wide and chromosome-wise significant SNPs and their nearest gene based on the GWAS.

TABLE S5 | GO enrichment analysis of the genes associated with the target SNPs at the chromosome-wise level as identified by the GWAS.

TABLE S6 | GO enrichments of the novel genes identified by the GWAS at the chromosome-wise level for the six sheep breeds, respectively.

TABLE S7 | Genotype effects of the most significant SNPs on litter size in six sheep breeds, respectively.

REFERENCES

- Aulchenko, Y. S., Ripke, S., Isaacs, A., and van Duijn, C. M. (2007). GenABEL: an R library for genome-wide association analysis. *Bioinformatics* 23, 1294–1296. doi: 10.1093/bioinformatics/btm108
- Bachelot, A., Monget, P., Imbert-Bollere, P., Coshigano, K., Kopchick, J. J., Kelly, P. A., et al. (2002). Growth hormone is required for ovarian follicular growth. *Endocrinology* 143, 4104–4112. doi: 10.1210/en.2002-220087
- Baird, D. T., Cnattingius, S., Collins, J., Evers, J. L. H., Glasier, A., Heitmann, B. L., et al. (2006). Nutrition and reproduction in women. *Hum. Reprod. Update* 12, 193–207. doi: 10.1093/humupd/dmk003
- Basini, G., Bussolati, S., Baioni, L., and Grasselli, F. (2011). Gelatinases (MMP2 and MMP9) in swine antral follicle. *Biofactors* 37, 117–120. doi: 10.1002/bi.0f.153
- Ben Sassi, N., Gonzalez-Recio, O., de Paz-del Rio, R., Rodriguez-Ramilo, S. T., and Fernandez, A. I. (2016). Associated effects of copy number variants on economically important traits in Spanish Holstein dairy cattle. *J. Dairy Sci.* 99, 6371–6380. doi: 10.3168/jds.2015-10487
- Bodin, L., Di Pasquale, E., Fabre, S., Bontoux, M., Monget, P., Persani, L., et al. (2007). A novel mutation in the bone morphogenetic protein 15 gene causing defective protein secretion is associated with both increased ovulation rate and sterility in Lacaune sheep. *Endocrinology* 148, 393–400. doi: 10.1210/en.2006-0764
- Bonferroni, C. E. (1936). Teoria statistica delle classi e calcolo delle probabilità. *Pubbl. R. Ist. Sup. Sci. Econ. Commer. Fir.* 8, 3–62.
- Cadoret, V., Frapsauce, C., Jarrier, P., Maillard, V., Bonnet, A., Locatelli, Y., et al. (2017). Molecular evidence that follicle development is accelerated in vitro compared to in vivo. *Reproduction* 153, 493–508. doi: 10.1530/Rep-16-0627
- Cao, J. X., Wei, C. H., Zhang, S. Z., Capellini, T. D., Zhang, L., Zhao, F. P., et al. (2016). Screening of reproduction-related single-nucleotide variations from MeDIP-seq data in sheep. *Mol. Reprod. Dev.* 83, 958–967. doi: 10.1002/mrd.22734
- Casas, E., Freking, B. A., and Leymaster, K. A. (2004). Evaluation of Dorset, Finnsheep, Romanov, Texel, and Montadale breeds of sheep: II. Reproduction of F1 ewes in fall mating seasons. *J. Anim. Sci.* 82, 1280–1289. doi: 10.2527/2004.8251280
- Castilho, A. C., Nogueira, M. F., Fontes, P. K., Machado, M. F., Satrapa, R. A., Razza, E. M., et al. (2014). Ovarian superstimulation using FSH combined with equine chorionic gonadotropin (eCG) upregulates mRNA-encoding proteins involved with LH receptor intracellular signaling in granulosa cells from Nelore cows. *Theriogenology* 82, 1199–1205. doi: 10.1016/j.theriogenology.2014.06.011
- Celik-Ozenci, C., Akkoyunlu, G., Kayisli, U. A., Arici, A., and Demir, R. (2003). Localization of vascular endothelial growth factor in the zona pellucida of developing ovarian follicles in the rat: a possible role in destiny of follicles. *Histochem. Cell Biol.* 120, 383–390. doi: 10.1007/s00418-003-0586-4
- Chang, H. M., Cheng, J. C., Klausen, C., and Leung, P. C. K. (2013). BMP15 suppresses progesterone production by down-regulating StAR via ALK3 in human granulosa cells. *Mol. Endocrinol.* 27, 2093–2104. doi: 10.1210/me.2013-1233
- Chen, X., Liu, Z., and Xu, J. (2010). The cooperative function of nuclear receptor coactivator 1 (NCOA1) and NCOA3 in placental development and embryo survival. *Mol. Endocrinol.* 24, 1917–1934. doi: 10.1210/me.2010-0201
- Chu, M. X., Jia, L. H., Zhang, Y. J., Jin, M., Chen, H. Q., Fang, L., et al. (2011). Polymorphisms of coding region of *BMP15* gene and their relationship with litter size in sheep. *Mol. Biol. Rep.* 38, 4071–4076. doi: 10.1007/s11033-010-0526-z
- Davis, G. H. (2004). Fecundity genes in sheep. *Anim. Reprod. Sci.* 82–83, 247–253. doi: 10.1016/j.anireprosci.2004.04.001
- Davis, G. H. (2005). Major genes affecting ovulation rate in sheep. *Genet. Sel. Evol.* 37, S11–S23. doi: 10.1051/gse:2004026
- Davis, G. H., Balakrishnan, L., Ross, I. K., Wilson, T., Galloway, S. M., Lumsden, B. M., et al. (2006a). Investigation of the Booroola (*FecB*) and Inverdale (*FecX1*) mutations in 21 prolific breeds and strains of sheep sampled in 13 countries. *Anim. Reprod. Sci.* 92, 87–96. doi: 10.1016/j.anireprosci.2005.06.001
- Davis, G. H., Farquhar, P. A., O'Connell, A. R., Everett-Hincks, J. M., Wishart, P. J., Galloway, S. M., et al. (2006b). A putative autosomal gene increasing ovulation rate in Romney sheep. *Anim. Reprod. Sci.* 92, 65–73.
- De Cesaro, M. P., Macedo, M. P., Santos, J. T., Rosa, P. R., Ludke, C. A., Rissi, V. B., et al. (2015). Natriuretic peptides stimulate oocyte meiotic resumption in bovine. *Anim. Reprod. Sci.* 159, 52–59. doi: 10.1016/j.anireprosci.2015.05.012
- de Souza, C. J. H., Mcneilly, A. S., Benavides, M. V., Melo, E. D. O., and Moraes, J. C. F. (2012). "Novel *GDF9* polymorphism determining higher ovulation rate and litter size in sheep," in *Proceedings of the Society for Reproduction and Fertility Annual Conference*, Edinburgh.
- Demars, J., Fabre, S., Sarry, J., Rossetti, R., Gilbert, H., Persani, L., et al. (2013). Genome-wide association studies identify two novel *BMP15* mutations responsible for an atypical hyperprolificacy phenotype in sheep. *PLoS Genet.* 9:e1003482. doi: 10.1371/journal.pgen.1003482
- Deniskova, T., Dotsev, A. V., Selionova, M., Wimmers, K., Reyer, H., Kharzinova, V. R., et al. (2017). Whole-genome single nucleotide polymorphism study of Romanov sheep. *J. Anim. Sci.* 95, 339–340. doi: 10.2527/asasann.2017.696
- Ding, N. S., Ren, D. R., Guo, Y. M., Ren, J., Yan, Y., Ma, J. W., et al. (2006). Genetic variation of porcine prostaglandin-endoperoxide synthase 2 (*PTGS2*) gene and

- its association with reproductive traits in an Erhualian × Duroc F2 population. *Yi Chuan Xue Bao* 33, 213–219. doi: 10.1016/S0379-4172(06)60042-5
- Drouilhet, L., Mansanet, C., Sarry, J., Tabet, K., Bardou, P., Woloszyn, F., et al. (2013). The highly prolific phenotype of Lacaune sheep is associated with an ectopic expression of the *B4GALNT2* gene within the ovary. *PLoS Genet.* 9:e1003809. doi: 10.1371/journal.pgen.1003809
- Eiriksson, J. H., and Sigurdsson, A. (2017). Sources of bias, genetic trend and changes in genetic correlation in carcass and ultrasound traits in the Icelandic sheep population. *Iceland. Agric. Sci.* 30, 3–12. doi: 10.16886/Ias.2017.01
- Fan, H. Y., O'Connor, A., Shitanaka, M., Shimada, M., Liu, Z., and Richards, J. S. (2010). Beta-catenin (CTNNB1) promotes preovulatory follicular development but represses LH-mediated ovulation and luteinization. *Mol. Endocrinol.* 24, 1529–1542. doi: 10.1210/me.2010-0141
- Feary, E. S., Juengel, J. L., Smith, P., French, M. C., O'Connell, A. R., Lawrence, S. B., et al. (2007). Patterns of expression of messenger RNAs encoding *GDF9*, *BMP15*, *TGFBR1*, *BMPRI1B*, and *BMPRI2* during follicular development and characterization of ovarian follicular populations in ewes carrying the Woodlands FecX2W mutation. *Biol. Reprod.* 77, 990–998. doi: 10.1095/biolreprod.107.062752
- Felsenstein, J. (1989). PHYLIP-phylogeny inference package (version 3.2). *Cladistics* 5, 164–166. doi: 10.1111/j.1096-0031.1989.tb00562.x
- Foroughinia, G., Fazileh, A., and Eghbalsaid, S. (2017). Expression of genes involved in BMP and estrogen signaling and AMPK production can be important factors affecting total number of antral follicles in ewes. *Theriogenology* 91, 36–43. doi: 10.1016/j.theriogenology.2016.12.023
- Galloway, S. M., McNatty, K. P., Cambridge, L. M., Laitinen, M. P., Juengel, J. L., Jokiranta, T. S., et al. (2000). Mutations in an oocyte-derived growth factor gene (*BMP15*) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nat. Genet.* 25, 279–283. doi: 10.1038/77033
- Haines, T. R., Rodenhiser, D. I., and Ainsworth, P. J. (2001). Allele-specific non-CpG methylation of the *Nf1* gene during early mouse development. *Dev. Biol.* 240, 585–598. doi: 10.1006/dbio.2001.0504
- Hanrahan, J. P., Gregan, S. M., Mulsant, P., Mullen, M., Davis, G. H., Powell, R., et al. (2004). Mutations in the genes for oocyte-derived growth factors *GDF9* and *BMP15* are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (*Ovis aries*). *Biol. Reprod.* 70, 900–909. doi: 10.1095/biolreprod.103.023093
- Hatzirodos, N., Hummitzsch, K., Irving-Rodgers, H. F., and Rodgers, R. J. (2015). Transcriptome comparisons identify new cell markers for theca interna and granulosa cells from small and large antral ovarian follicles. *PLoS One* 10:e0119800. doi: 10.1371/journal.pone.0119800
- Huang, D. W., Sherman, B. T., and Lempicki, R. A. (2009a). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* 4, 44–57. doi: 10.1038/nprot.2008.211
- Huang, D. W., Sherman, B. T., and Lempicki, R. A. (2009b). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* 37, 1–13. doi: 10.1093/nar/gkn923
- Huang, H. Y., Li, S. F., Zhao, Z. H., Liang, Z., Zhang, J., and Ding, Y. R. (2011). Association of polymorphisms for nuclear receptor coactivator 1 gene with egg production traits in the maternal line of Shaobo hens. *Br. Poult. Sci.* 52, 328–332. doi: 10.1080/00071668.2011.577057
- Hu, X. J., Pokharel, K., Peippo, J., Ghanem, N., Zhaboyev, I., Kantanen, J., et al. (2016). Identification and characterization of miRNAs in the ovaries of a highly prolific sheep breed. *Anim. Genet.* 47, 234–239. doi: 10.1111/age.12385
- Kang, H. M., Sul, J. H., Service, S. K., Zaitlen, N. A., Kong, S. Y., Freimer, N. B., et al. (2010). Variance component model to account for sample structure in genome-wide association studies. *Nat. Genet.* 42, 348–354. doi: 10.1038/ng.548
- Knapp, E., and Sun, J. J. (2017). Steroid signaling in mature follicles is important for *Drosophila* ovulation. *Proc. Natl. Acad. Sci. U.S.A.* 114, 699–704. doi: 10.1073/pnas.1614383114
- Köchl, S., Niederstätter, H., and Parson, W. (2005). DNA extraction and quantitation of forensic samples using the phenol-chloroform method and real-time PCR. *Forensic DNA Typing Protoc.* 297, 13–30. doi: 10.1385/1-59259-867-6:013
- Kumar, M., Camlin, N. J., Holt, J. E., Teixeira, J. M., McLaughlin, E. A., and Tanwar, P. S. (2016). Germ cell specific overactivation of WNT/beta catenin signalling has no effect on folliculogenesis but causes fertility defects due to abnormal foetal development. *Sci. Rep.* 6:27273. doi: 10.1038/srep27273
- Laliotis, G. P., Marantidis, A., and Avdi, M. (2017). Association of *BF*, *RBP4*, and *ESR2* genotypes with litter size in an autochthonous pig population. *Anim. Biotechnol.* 28, 138–143. doi: 10.1080/10495398.2016.1242490
- Lassoued, N., Benkhilil, Z., Woloszyn, F., Rejeb, A., Aouina, M., Rekik, M., et al. (2017). FecX(Bar) a novel *BMP15* mutation responsible for prolificacy and female sterility in Tunisian Barbarine sheep. *BMC Genet.* 18:43. doi: 10.1186/s12863-017-0510-x
- Martinez-Royo, A., Jurado, J. J., Smulders, J. P., Marti, J. I., Alabart, J. L., Roche, A., et al. (2008). A deletion in the *bone morphogenetic protein 15* gene causes sterility and increased prolificacy in Rasa Aragonesa sheep. *Anim. Genet.* 39, 294–297. doi: 10.1111/j.1365-2052.2008.01707.x
- Miao, X., Luo, Q., Zhao, H., and Qin, X. (2016). Ovarian transcriptomic study reveals the differential regulation of miRNAs and lncRNAs related to fecundity in different sheep. *Sci. Rep.* 6:35299. doi: 10.1038/srep35299
- Monteagudo, L. V., Ponz, R., Tejedor, M. T., Lavina, A., and Sierra, I. (2009). A 17 bp deletion in the Bone Morphogenetic Protein 15 (*BMP15*) gene is associated to increased prolificacy in the Rasa Aragonesa sheep breed. *Anim. Reprod. Sci.* 110, 139–146. doi: 10.1016/j.anireprosci.2008.01.005
- Moradband, F., Rahimi, G., and Gholizadeh, M. (2011). Association of polymorphisms in fecundity genes of *GDF9*, *BMP15* and *BMP15-1B* with litter size in Iranian Baluchi sheep. *Asian Australas. J. Anim. Sci.* 24, 1179–1183. doi: 10.5713/ajas.2011.10453
- Mullen, M. P., and Hanrahan, J. P. (2014). Direct evidence on the contribution of a missense mutation in *GDF9* to variation in ovulation rate of Finnish. *PLoS One* 9:e95251. doi: 10.1371/journal.pone.0095251
- Mulsant, P., Lecerf, F., Fabre, S., Schibler, L., Monget, P., Lanneluc, I., et al. (2001). Mutation in bone morphogenetic protein receptor-IB is associated with increased ovulation rate in Booroola Merino ewes. *Proc. Natl. Acad. Sci. U.S.A.* 98, 5104–5109. doi: 10.1073/pnas.091577598
- Munoz, G., Ovilo, C., Estelle, J., Silio, L., Fernandez, A., and Rodriguez, C. (2007). Association with litter size of new polymorphisms on *ESR1* and *ESR2* genes in a Chinese-European pig line. *Genet. Sel. Evol.* 39, 195–206. doi: 10.1186/1297-9686-39-2-195
- Nicol, L., Bishop, S. C., Pong-Wong, R., Bendixen, C., Holm, L. E., Rhind, S. M., et al. (2009). Homozygosity for a single base-pair mutation in the oocyte-specific *GDF9* gene results in sterility in Thoka sheep. *Reproduction* 138, 921–933. doi: 10.1530/REP-09-0193
- Ogiwara, K., and Takahashi, T. (2017). Involvement of the nuclear progesterin receptor in LH-induced expression of membrane type 2-matrix metalloproteinase required for follicle rupture during ovulation in the medaka, *Oryzias latipes*. *Mol. Cell. Endocrinol.* 450, 54–63. doi: 10.1016/j.mce.2017.04.016
- Patterson, N., Price, A. L., and Reich, D. (2006). Population structure and eigenanalysis. *PLoS Genet.* 2:e190. doi: 10.1371/journal.pgen.0020190
- Peng, W. F., Xu, S. S., Ren, X., Lv, F. H., Xie, X. L., Zhao, Y. X., et al. (2017). A genome-wide association study reveals candidate genes for the supernumerary nipple phenotype in sheep (*Ovis aries*). *Anim. Genet.* 48, 570–579. doi: 10.1111/age.12575
- Petryszak, R., Keays, M., Tang, Y. A., Fonseca, N. A., Barrera, E., Burdett, T., et al. (2016). Expression Atlas update-an integrated database of gene and protein expression in humans, animals and plants. *Nucleic Acids Res.* 44, D746–D752. doi: 10.1093/nar/gkv1045
- Poole, D. H., Ndiaye, K., and Pate, J. L. (2013). Expression and regulation of secreted phosphoprotein 1 in the bovine corpus luteum and effects on T lymphocyte chemotaxis. *Reproduction* 146, 527–537. doi: 10.1530/Rep-13-0190
- Price, A. L., Patterson, N. J., Plenge, R. M., Weinblatt, M. E., Shadick, N. A., and Reich, D. (2006). Principal components analysis corrects for stratification in genome-wide association studies. *Nat. Genet.* 38, 904–909. doi: 10.1038/ng1847
- Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M. A., Bender, D., et al. (2007). PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.* 81, 559–575. doi: 10.1086/519795
- Putnova, L., Kolarikova, O., Knoll, A., and Dvorák, J. (2001). Association study of osteopontin (*SPPI*) and estrogen receptor (*ESR*) genes with reproduction traits in pigs. *Acta Univ. Agric. Silv. Mendel. Brun.* 49, 69–74.

- Raymond, M., and Rousset, F. (1995). Genepop (version-1.2)-population-genetics software for exact tests and ecumenicism. *J. Hered.* 86, 248–249. doi: 10.1093/oxfordjournals.jhered.a111573
- Regan, S. L., McFarlane, J. R., O'Shea, T., Andronicos, N., Arfuso, F., Dharmarajan, A., et al. (2015). Flow cytometric analysis of *FSHR*, *BMRR1B*, *LHR* and apoptosis in granulosa cells and ovulation rate in merino sheep. *Reproduction* 150, 151–163. doi: 10.1530/Rep-14-0581
- Rumi, M. A. K., Singh, P., Roby, K. F., Zhao, X., Iqbal, K., Ratri, A., et al. (2017). Defining the role of estrogen receptor beta in the regulation of female fertility. *Endocrinology* 158, 2330–2343. doi: 10.1210/en.2016-1916
- Sayasith, K., Sirois, J., and Lussier, J. G. (2014). Expression and regulation of regulator of G-protein signaling protein-2 (*RGS2*) in equine and bovine follicles prior to ovulation: molecular characterization of *RGS2* transactivation in bovine granulosa cells. *Biol. Reprod.* 91, 75–86. doi: 10.1095/biolreprod.114.121186
- Silva, B. D. M., Castro, E. A., Souza, C. J. H., Paiva, S. R., Sartori, R., Franco, M. M., et al. (2011). A new polymorphism in the growth and differentiation factor 9 (*GDF9*) gene is associated with increased ovulation rate and prolificacy in homozygous sheep. *Anim. Genet.* 42, 89–92. doi: 10.1111/j.1365-2052.2010.02078.x
- Souza, C. J. H., MacDougall, C., Campbell, B. K., McNeilly, A. S., and Baird, D. T. (2001). The Booroola (FecB) phenotype is associated with a mutation in the bone morphogenetic receptor type 1 B (*BMPRI1B*) gene. *J. Endocrinol.* 169, R1–R6. doi: 10.1677/joe.0.169R001
- Szklarczyk, D., Morris, J. H., Cook, H., Kuhn, M., Wyder, S., Simonovic, M., et al. (2017). The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Res.* 45, D362–D368. doi: 10.1093/nar/gkw937
- Tal, R., Seifer, D. B., Grazi, R. V., and Malter, H. E. (2014). Follicular fluid placental growth factor is increased in polycystic ovarian syndrome: correlation with ovarian stimulation. *Reprod. Biol. Endocrinol.* 12, 1–7. doi: 10.1186/1477-7827-12-82
- Tang, H., Liu, Y., Li, J., Li, G., Chen, Y., Yin, Y., et al. (2017). LH signaling induced *ptgs2a* expression is required for ovulation in zebrafish. *Mol. Cell. Endocrinol.* 447, 125–133. doi: 10.1016/j.mce.2017.02.042
- Terenina, E., Fabre, S., Bonnet, A., Monniaux, D., Robert-Granie, C., SanCristobal, M., et al. (2017). Differentially expressed genes and gene networks involved in pig ovarian follicular atresia. *Physiol. Genomics* 49, 67–80. doi: 10.1152/physiolgenomics.00069.2016
- Vage, D. I., Husdal, M., Kent, M. P., Klemetsdal, G., and Boman, I. A. (2013). A missense mutation in growth differentiation factor 9 (*GDF9*) is strongly associated with litter size in sheep. *BMC Genet.* 14:1. doi: 10.1186/1471-2156-14-1
- Vastagh, C., Rodolose, A., Solymosi, N., and Liposits, Z. (2016). Altered expression of genes encoding neurotransmitter receptors in GnRH neurons of proestrous mice. *Front. Cell. Neurosci.* 10:230. doi: 10.3389/Fncel.2016.00230
- Vo, K. K., Jarocho, D. J., Lyde, R. B., Hayes, V., Thom, C. S., Sullivan, S. K., et al. (2017). *FLI1* level during megakaryopoiesis affects thrombopoiesis and platelet biology. *Blood* 129, 3486–3494. doi: 10.1182/blood-2017-02-770958
- Wilson, A. J., Pemberton, J. M., Pilkington, J. G., Clutton-Brock, T. H., and Kruuk, L. E. (2009). Trading offspring size for number in a variable environment: selection on reproductive investment in female Soay sheep. *J. Anim. Ecol.* 78, 354–364. doi: 10.1111/j.1365-2656.2008.01489.x
- Wilson, T., Wu, X. Y., Juengel, J. L., Ross, I. K., Lumsden, J. M., Lord, E. A., et al. (2001). Highly prolific Booroola sheep have a mutation in the intracellular kinase domain of bone morphogenetic protein IB receptor (*ALK-6*) that is expressed in both oocytes and granulosa cells. *Biol. Reprod.* 64, 1225–1235. doi: 10.1095/biolreprod64.4.1225
- Xiao, X. H., Liu, A. Q., Wen, H. X., Tian, Y. J., Ni, J., and Liu, G. Y. (2009). Expression and localization of transcription factor Ets-1 in the rat ovary during the estrous cycle and pregnancy. *Fertil. Steril.* 91, 1998–2005. doi: 10.1016/j.fertnstert.2008.02.166
- Xu, S.-S., and Li, M.-H. (2017). Recent advances in understanding genetic variants associated with economically important traits in sheep (*Ovis aries*) revealed by high-throughput screening technologies. *Front. Agr. Sci. Eng.* 4, 279–288. doi: 10.15302/J-FASE-2017151
- Xu, Y. F., Li, E. L., Han, Y. D., Chen, L., and Xie, Z. A. (2010). Differential expression of mRNAs encoding BMP/Smad pathway molecules in antral follicles of high- and low-fecundity Hu sheep. *Anim. Reprod. Sci.* 120, 47–55. doi: 10.1016/j.anireprosci.2010.02.009
- Yue, G. H. (1996). Reproductive characteristics of Chinese Hu sheep. *Anim. Reprod. Sci.* 44, 223–230. doi: 10.1016/0378-4320(96)01562-X
- Zhai, B., Liu, H. Y., Li, X., Dai, L. S., Gao, Y., Li, C. H., et al. (2013). BMP15 prevents cumulus cell apoptosis through CCL2 and FBN1 in porcine ovaries. *Cell. Physiol. Biochem.* 32, 264–278. doi: 10.1159/000354435
- Zhang, C. S., Geng, L. Y., Du, L. X., Liu, Z. Z., Fu, Z. X., Feng, M. S., et al. (2011). Polymorphic study of FecX(G), FecG(H) and Fec(B) mutations in four domestic sheep breeds in the Lower Yellow River Valley of China. *J. Anim. Vet. Adv.* 10, 2198–2201. doi: 10.3923/javaa.2011.2198.2201

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Xu, Gao, Xie, Ren, Shen, Wang, Shen, Eyþórsdóttir, Hallsson, Kiseleva, Kantanen and Li. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.