Polymorphism, expression and structure analysis of key genes in the ovarian steroidogenesis pathway in sheep (*Ovis aries*)

Wen-Ping Hu¹ | Ming-Qiu Liu¹,² | Zhi-Long Tian¹ | Qiu-Yue Liu³ | Zhuang-Biao Zhang¹ | Ji-Shun Tang¹,⁴ | Xiao-Yun He¹ | Yan-Yan Zhu² | Yuan-Yuan Wang² | Ming-Xing Chu¹

¹Key Laboratory of Animal Genetics and Breeding and Reproduction of Ministry of Agriculture and Rural Affairs, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, China
²Department of Biology Science, Bengbu Medical College, Bengbu, China
³Institute of Genetics and Developmental Biology, The Innovation Academy for Seed Design, Chinese Academy of Sciences, Beijing, China
⁴Institute of Animal Husbandry and Veterinary Medicine, Anhui Academy of Agricultural Sciences, Hefei, China

Correspondence
Yuan-Yuan Wang, Department of Biology Science, Bengbu Medical College, Bengbu 233030, China.
Email: nlicau@163.com

Ming-Xing Chu, Key Laboratory of Animal Genetics and Breeding and Reproduction of Ministry of Agriculture and Rural Affairs, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, China.
Email: mxchu@263.net

Funding information
National Natural Science Foundation of China, Grant/Award Number: 31501941 and 31772580; Central Public-interest Scientific Institution Basal Research Fund for Chinese Academy of Tropical Agricultural Sciences, Grant/Award Number: 2018-YWF-YB-1 and Y2017JC24; Genetically Modified Organisms Breeding Major Program of China, Grant/Award Number: 2016ZX08009-003-006; Earmarked Fund for China Agriculture Research System, Grant/Award Number: CARS-38; Agricultural Science and Technology Innovation Program of China, Grant/Award Number: ASTIP-IAS13

Abstract

**Background:** Litter size is an important factor that significantly affects the development of the sheep industry. Our previous TMT proteomics analysis found that three key proteins in the ovarian steroidogenesis pathway, STAR, HSD3B1, and CYP11A1, may affect the litter size trait of Small Tail Han sheep.

**Objective:** The purpose of this study was to better understand the relationship between polymorphisms of these three genes and litter size.

**Material and Method:** Sequenom MassARRAY detected genetic variance of the three genes in 768 sheep. Real-time qPCR of the three genes was used to compare their expression in monotocous and polytocous sheep in relevant tissues. Finally, bioinformatics analysis predicted the protein sequences of the different SNP variants.

**Result:** Association analysis showed that there was a significant difference in litter size among the genotypes at two loci of the *CYP11A1* gene (p < 0.05), but no significant difference was observed in litter size among all genotypes at all loci of the *STAR* and *HSD3B1* genes (p > 0.05). However, STAR expression was significantly different in polytocous and monotonous sheep in the pituitary (p < 0.01). Tissue-specific expression in the ovary was observed for *HSD3B1* (p < 0.05), but its expression was not different between polytocous and monotonous sheep. Bioinformatics analysis showed that the g.33217408C > T mutation of *CYP11A1* resulted in major changes to the secondary and tertiary structures. In contrast, gene polymorphisms in *STAR* and *HSD3B1* had minimal impacts on their protein structures.

**Discussion:** This may explain why the CYP11A1 variant impacted litter size while the others did not. The single nucleotide polymorphism of the CYP11A1 gene would
serve as a good molecular marker when breeding to increase litter size in sheep. Our study provides a basis for further revealing the function of the ovarian steroidogenesis pathway in sheep reproduction and sheep breeding.

**KEYWORDS**
CYP11A1, HSD3B1, litter size, ovarian steroidogenesis pathway, sheep, STAR

## 1 | INTRODUCTION

Since the domestication of sheep, their various products have become important staples in society, including their meat, wool, skin, milk and others. Among them, mutton is an important source of protein for humans. Mutton sheep production plays an important role in the animal husbandry economy. The sheep industry prioritizes three traits to maximize economic benefits: reproduction, meat production and fur. Of these, improved reproductive traits yield the most gains in revenue. The economic benefit of double lambing is more than 1.6 times that of single lambing (Notter, 2012). There are 700 sheep breeds in the world; the 71 in China are comprised of 42 local breeds, eight introduced breeds and 21 cultivated breeds. Only a few breeds consistently birth more than one sheep at a time; these so-called polytocous sheep have a lambing rate of more than 200%. Statistically, most species of sheep are seasonal oestrus and monotocous—generally yielding only one lamb per litter per year. There are some famous sheep breeds with a lambing rate of 200% or above, including Booroola Merino, Inverdale, Cambridge, Thoka, Javanese, Olkuska, Belclare, Lacaune and Woodlands sheep, as well as Hu sheep and Small Tail Han sheep in China (Zheng et al., 2019). The Booroola Merino average lambing rate can reach 350%, while the average lambing rate of Small Tail Han sheep and Hu sheep are above 267.1% and 277.4%, respectively. Despite their lower lambing rate, these Chinese varieties have more consistent performance in lamb production, a result of long-term natural selection and artificial selection (National Committee on Livestock and Poultry Genetic Resources, 2012). Reproduction is extremely complicated; environmental (Greives et al., 2007), nutritional (Abecia et al., 2015) and genetic factors (McLaren, 1998) all play an important role in the regulation of sheep reproductive processes.

In 1982, Davis et al. identified the main gene controlling litter size in Booroola Merino sheep in Australia (Davis et al., 1982). In 1989, it was officially named FecB (Fec = fecundity, B = Booroola). Since then, the search for other major genes has become the goal of sheep geneticists. Small Tail Han is a famous polytocous sheep breed from China (Tang et al., 2019). This breed has three different FecB genotypes (BB, B+ and +/+). In our previous TMT proteomics study, the ovarian steroidogenesis pathway was significantly enriched (according to KEGG pathway analysis) in the ovaries of Small Tail Han sheep lacking the FecB mutation (FecB+__). It led us to hypothesize that the key proteins of the ovarian steroidogenesis pathway, STAR (steroidogenic acute regulatory protein), HSD3B1 (hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1) and CYP11A1 (Cytochrome P450 family 11 subfamily A member 1), affect litter size (Tang et al., 2019).

Steroid hormones control metabolism, inflammation, immune functions, salt and water balance, development of sexual characteristics, and the ability to withstand illness and injury (Busada & Cidlowski, 2017; Frye, 2009; Fujita, 2010). In terms of pharmacological action, there are three types of steroid hormones, corticosteroid, sex hormone and mineralocorticoid. Sex hormones are further subdivided into male and female hormones. They are related to sex and the development of secondary sexual characteristics in animals. Oestrogen is found in all vertebrates and is responsible for the maturation and maintenance of the vagina and uterus. It is also involved in ovarian functions, such as the maturation of ovarian follicles. Oestrogen also plays an important role in the regulation of gonadotropin secretion (Baker, 2013; Nelson & Bulun, 2001). Progestogens are another type of steroid hormones that are secreted by luteal cells in the ovary. Their main physiological functions support a safe pregnancy: they inhibit ovulation, promote endometrium secretion, facilitate fertilized egg implantation and reduce uterine muscle excitement (Haas & Ramsey, 2013; Wahabi et al., 2018). Thus, steroid hormones are necessary for successful reproduction in females. The ovarian steroidogenesis pathway is closely related to ovarian function and follicular development (Gareis et al., 2018). This pathway includes STAR, HSD3B1, CYP1A1, CYP1B1, CYP11A1, CYP17A1 and CYP19A1. These genes encode the enzymes that synthesize steroids in the ovary. Steroidogenic acute regulatory protein, encoded by the STAR gene, mediates the transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, where lysis to gestenolone occurs. Thus, STAR indirectly increases the progesterone levels in the body that affect ovulation and reproductive functions (Di Renzo et al., 2016; Hanukoglu et al., 1977; Kohen et al., 2003; Tajima et al., 2003; Vallee, 2016). HSD3B1 encodes 3β-hydroxyl steroid dehydrogenase/isomerase (3β-HSD), which plays a crucial role in the biosynthesis of all hormonal steroids. This enzyme affects ovulation by converting pregnenolone to progesterone (Koritz, 1964). The proteins encoded by CYP1A1, CYP1B1, CYP11A1, CYP17A1 and CYP19A1 are monoxygenases in the cytochrome P450 superfamily. In sheep and other mammals, these proteins are important for the removal of xenobiotics, as well as the synthesis and breakdown of endogenous hormones, including oxidized steroids, and fatty acids (Danielson, 2002; Gonzalez & Gelboin, 1992). Cytochrome
P450 side chain lyase (P450scC) encoded by the CYP11A1 gene is a mitochondrial enzyme that plays an important role in the regulation of the catalytic conversion of cholesterol to progesterone (O’Hara et al., 2014).

The STAR, HSD3B1 and CYP11A1 genes of the ovarian steroidogenesis pathway were directly involved in the synthesis of the steroid hormone, progesterone. Our previous quantitative mass spectrometry data revealed an association between these proteins and the polytocous trait. But there were no relevant studies on the correlation between STAR, HSD3B1 and CYP11A1 genes with the litter size of sheep. To better understand the effects of these three genes, Sequenom MassARRAY technology was used to detect gene polymorphisms in seven sheep breeds. The association between polymorphism of these genes and litter size was analysed. The expression levels of these genes were also measured in Small Tail Han ewes, in order to understand the relationship between gene expression and litter size. Our findings could provide a reference for further research on molecular marker-assisted breeding of sheep.

2 | MATERIALS AND METHODS

2.1 | Blood sample collection and DNA extraction

Jugular blood samples (10-ml blood per sheep) from 768 sheep of seven breeds were collected into EDTA-coated tubes. Genomic DNA was extracted using blood genomic DNA extraction kits (TIANGEN Biotech Co., Ltd.) and stored at −20°C. The isolated DNA was measured by agarose gel electrophoresis to ensure its quality.

Small Tail Han sheep (n = 384), Cele Black sheep (n = 68), and Hu sheep (n = 83) were the polytocous sheep breeds in this study. Tan sheep (n = 23), Prairie Tibetan (n = 80), Suffolk sheep (n = 60) and Sunite sheep (n = 70) were the monotocous sheep breeds in this study. The selected sheep were of similar age and fed in an indoor setting under similar conditions of room temperature, illumination, feeding system and nutrition level.

2.2 | Genotyping

According to the early re-sequencing data (version 4.1) from our laboratory consisting of 100 individual sheep from 10 different breeds (Pan et al., 2018), SNP loci of the STAR, HSD3B1 and CYP11A1 genes were screened. The g.31959747T>C, g.31962677G>A, g.31959875C>A and g.31960614C>T loci in the STAR gene; g.96094239C>T, g.96095151G>A, g.96101288G>A, g.96092576G>A and g.96101542C>A loci in the HSD3B1 gene; and g.33222725A>G and g.33217408C>T loci in the CYP11A1 gene were selected for genotyping in 768 polytocous and monocotous sheep, including Small Tail Han sheep, Cele Black sheep, Hu sheep, Tan sheep, Prairie Tibetan sheep, Suffolk sheep and Sunite sheep using MassARRAY (Zhang et al., 2019). First, the invariant region of each gene was amplified. This initial amplification was then subjected to single-base extended PCR using specific primers. The molecular weight of products after extension, which differs due to incorporation of different bases at polymorphic sites, were analysed by time-of-flight mass spectrometry to identify the different genotypes (Zhang et al., 2019).

2.3 | Statistical analysis

Allele frequencies, genotype frequencies, p values, polymorphism information content (PIC), heterozygosity (HE) and the 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. A linear model using the equation \( \Delta y_{ij} = \mu + P_i + G_j + I_{PG} + e_{ijn} \) was subsequently applied to analyse the association of genotypes and litter size, in which \( \Delta y_{ij} \) represents the phenotypic value (litter size); \( \mu \) is the population mean; \( P_i \) is 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.

2.4 | Gene expression in the reproductive axis of sheep

Small Tail Han sheep were selected randomly from the polytocous and monocotous groups. The tissue samples of this experiment were collected from a farm at the Tianjin Animal Husbandry and Veterinary Research Institute. The RNA was extracted from the hypothalamus, pituitary and ovary tissues according to the instructions of the RNA extraction kits (TIANGEN Biotech Co., Ltd.). Primer Premier 6.0 and Beacon Designer 8.0 were used for to design primers for STAR, HSD3B1 and CYP11A1, and the internal reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using sequences from GenBank.

Reverse transcription PCR (RT-PCR) was performed using the PrimeScript™ RT Enzyme Mix II, 1-μl Oligo dT Primer, 1-μl Random 6 mers, 4-μl 5X PrimeScript Buffer (for Real-Time), 1-μg RNA, and RNase-free ddH₂O. The reaction conditions of RT-PCR were: 37°C for 15 min and 85°C for 5 s. The 20-μl quantitative real-time PCR (RT-qPCR) reaction system contained: 10.0-μl SYBR Premix Ex Taq II, 0.8-μl upstream and downstream primers, 2.0-μl cDNA template, and 6.4-μl RNase-free ddH₂O. The PCR procedure started with pre-denaturation at 95°C for 5 s, followed by 40 cycles of denaturation at 95°C for 5 s and denaturation at 60°C for 30 s. To determine relative expression, the CT value of each gene in Small Tail Han sheep hypothalamic, pituitary and ovary tissues was obtained by RT-qPCR. Next, the ΔCT was calculated as CT value–average CT value of GAPDH, then the ΔΔCT value was calculated as the ΔCT value–average ΔCT value of GAPDH. Finally, the relative expression was represented as \( 2^{-\Delta\Delta CT} \).
2.5 | Bioinformatics analysis

First, the sequences of the genes were obtained from NCBI (https://www.ncbi.nlm.nih.gov/), and the amino acid sequences were obtained from UniProt (https://www.uniprot.org/). Prediction of the secondary structure of each gene and its variants was carried out using PredictProtein (https://www.predictprotein.org/). The three-dimensional (3-D) structures before and after mutation were predicted with Phyre2 (Kelley et al., 2015). The transmembrane domains before and after mutation were predicted using TMHMM (Krogh et al., 2001).

3 | RESULTS

3.1 | Population genetic analysis of known SNPs in STAR, HSD3B1 and CYP11A1 genes

The SNPs identified in STAR, HSD3B1, and CYP11A1 genes are listed in Table 1. Each of the three possible genotypes at the g.31959747T>C and g.31962677G>A loci of the STAR gene (Table 2), and at the g.96094239C>T and g.96101542C>A loci of the HSD3B1 gene was detected in the population of tested sheep (Table 3). However, only two genotypes were detected at the SNP loci of CYP11A1 (Table 4). The data showed that only the g.31962677G>A locus of the STAR gene and the g.96094239C>T locus of the HSD3B1 gene was moderately polymorphic (0.25 < PIC < 0.5) in all sheep breeds. The g.31959875C>A locus of the STAR gene was moderately polymorphic (0.25 < PIC < 0.5) in Prairie Tibetan sheep and Suffolk sheep. The g.96101542C>A locus of the HSD3B1 gene was moderately polymorphic (0.25 < PIC < 0.5) in Small Tail Han sheep, Cele Black sheep and Hu sheep. The other loci all had a low-degree polymorphism (PIC < 0.25). According to the available data, the g.31959747T>C locus was not in Hardy–Weinberg equilibrium (p < 0.05) in Cele Black sheep, Hu sheep, Suffolk sheep and Sunite sheep; the g.31959875T>C locus was not in Hardy–Weinberg equilibrium (p < 0.05) in Prairie Tibetan sheep and Suffolk sheep; and the g.96101542T>C locus was not in Hardy–Weinberg equilibrium (p < 0.05) in Suffolk sheep. All other populations were in Hardy–Weinberg equilibrium (p > 0.05).

3.2 | Correlation analysis between SNPs and litter size of Small Tail Han sheep

In order to demonstrate the correlation between SNPs and litter size of sheep more intuitively, we carried out association analysis between the number of individuals of each genotype and litter size (Table 5). The result of litter size is expressed as the average with standard deviation. No significant difference in litter size between different genotypes of both the STAR and HSD3B1 genes was found (p > 0.05). However, there was a significant difference in litter size between different genotypes of the CYP11A1 gene (p < 0.05). The g.33222725A>G locus was associated with litter size at all parities, and sheep with the AA genotype had a greater litter size than those with the AG genotype (p < 0.05). The g.33217408C>T SNP also correlated with litter size at all parities, and sheep with the CT genotype had a greater litter size than those with CC genotype (p < 0.05).

3.3 | Gene expression in the reproductive axis of sheep

Gene expression in hypothalamic, pituitary and ovary tissues of Small Tail Han sheep was studied between monotocous and polytocous populations by RT-qPCR (Figure 1). The STAR gene was expressed in all three tissues, and there was a significant difference between polytocous sheep and monotocous sheep in the pituitary (p < 0.01). No significant difference between polytocous sheep and monotocous sheep of the HSD3B1 gene in the ovary was found (p > 0.05). But the HSD3B1 gene was mainly expressed in ovary, and not in the other two tissues (p < 0.05). According to our previous study (Tian et al., 2019), the CYP11A1 gene is also mainly expressed in ovary, and

| Gene | SNP position | Consequence type | Nucleotide change | Codon change | Amino acid change |
|------|--------------|-----------------|------------------|-------------|-----------------|
| STAR | g.31959747T | 3' UTR variant | T>C | — | — |
|      | g.31962677G | CYP11A1 missense variant | G>A | GCC/GTC | A/V |
|      | g.31959875T | 3' UTR variant | C>A | — | — |
|      | g.31960614C | missense variant | C>T | CGC/CAC | R/H |
| HSD3B1 | g.96094239C | intron variant | C>T | — | — |
|      | g.96095151A | downstream variant | G>A | — | — |
|      | g.96101288G | missense variant | G>A | CGG/CAG | R/Q |
|      | g.96092576G | missense variant | G>A | GCC/ACC | A/T |
|      | g.96101542C | missense variant | C>A | CTG/ATG | L/M |
| CYP11A1 | g.33222725A | synonymous variant | A>G | TCA/TGC | — |
|      | g.33217408T | missense variant | C>T | TCC/TTC | S/F |

TABLE 1 | SNPs observed in STAR, HSD3B1 and CYP11A1 genes
its expression in monotocous sheep is significantly lower than that in polytocous sheep ($p < 0.05$).

### 3.4 Bioinformatics analysis

Several SNPs resulted in amino acid changes (Table 1). In the STAR gene, SNPs g.31959747T>C and g.31960614C>T resulted in amino acid changes at residue 103 (Ala to Val) and residue 274 (Arg to His), respectively. In HSD3B1, SNPs g.96101288 G>A, g.96092576 G>A and g.96101542 C>A caused amino acid changes at residue 285 (Arg to Gln), residue 2 (Ala to Thr) and residue 370 (Leu to Met. SNP g.33217408C>T of CYP11A1 caused an amino acid change at residue 41 (Ser to Phe).

The effects of these amino acid changes on the protein secondary structure was predicted by PredictProtein (Kelley et al., 2015). For the STAR protein, the three protein-binding regions in proximity to amino acids 37, 274 and 281 (Figure 2a) were no longer present after simultaneous mutations at g.31962677G>A and g.31960614C>T, and there was a new protein-binding region in proximity to amino acid 192 (Figure 2b). HSD3B1 had three protein-binding regions near amino acid numbers 45, 92 and 284 (Figure 2c), but after mutations at g.96101288G>A, g.96092576G>A and g.96101542C>A, they were absent (Figure 2d). For CYP11A1, there

| TABLE 2 Polymorphism of STAR gene and population genetic analysis in seven sheep breeds |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Breed                             | Number     | Genotype frequency | Allele frequency | PIC  | HE     | NE     | Chi-square test (p value) |
|----------------------------------|------------|--------------------|-------------------|------|--------|--------|---------------------------|
| Small Tail Han                    | 378        | 0.01 (2)           | 0.02 (8)          | 0.97 (368) | 0.02 | 0.98  | 0.03  | 0.03  | 1.03 (0.73) |
| Cele Black                        | 68         | 0.01 (1)           | 0.01 (1)          | 0.98 (66)  | 0.02 | 0.98  | 0.03  | 0.03  | 1.03 (0.00) |
| Hu                               | 83         | 0.06 (5)           | 0.08 (7)          | 0.86 (71)  | 0.10 | 0.90  | 0.16  | 0.18  | 1.22 (0.00) |
| Tan                              | 23         | 0.04 (1)           | 0.00 (0)          | 0.96 (22)   | 0.04 | 0.96  | 0.07  | 0.08  | 1.08 (0.00) |
| Prairie Tibetan                  | 80         | 0.03 (2)           | 0.16 (13)         | 0.81 (65)   | 0.11 | 0.89  | 0.18  | 0.20  | 1.24 (0.20) |
| Suffolk                          | 60         | 0.02 (1)           | 0.02 (1)          | 0.96 (58)   | 0.03 | 0.97  | 0.06  | 0.06  | 1.06 (0.00) |
| Sunite                           | 70         | 0.09 (6)           | 0.04 (3)          | 0.87 (61)   | 0.11 | 0.89  | 0.18  | 0.20  | 1.24 (0.00) |

Abbreviations: HE, heterozygosity; NE, number of effective alleles; PIC, polymorphism information content.
was an RNA-binding region in proximity to amino acid number 43, one protein-binding region near amino acid 143, and one DNA-binding region near amino acid 459 (Figure 2e); these binding sites were no longer present after the g.33217408C>T mutation, but two new protein-binding regions in proximity to amino acids 119 and 352, and one DNA-binding region in proximity to amino acid 46 were present after the g.33217408C>T mutation (Figure 2f). Unlike STAR and HSD3B1 proteins, CYP11A1 had only one SNP

### TABLE 3 Polymorphism of the HSD3B1 gene and population genetic analysis in seven sheep breeds

| Breed                  | Number | Genotype frequency | Allele frequency | PIC  | HE  | NE  | Chi-square test (p value) |
|------------------------|--------|--------------------|------------------|------|-----|-----|---------------------------|
|                        |        | CC                | CT               | TT   | C   | T   |                           |
| g.96094239C>T          |        | 0.11 (44)         | 0.41 (157)       | 0.48 (182) | 0.32 | 0.68 | 0.34 | 0.44 | 1.77 | 0.26 |
|                        | Small Tail Han | 383               |                  |      |     |     |                           |
|                        | Cele Black    | 68                |                  |      |     |     |                           |
|                        | Hu            | 82                |                  |      |     |     |                           |
|                        | Tan           | 23                |                  |      |     |     |                           |
|                        | Prairie Tibetan | 80              |                  |      |     |     |                           |
|                        | Suffolk       | 60                |                  |      |     |     |                           |
|                        | Sunite        | 70                |                  |      |     |     |                           |
| g.96095151G>A          |        | 1.00 (383)        | 0.00 (0)         | 0.00 (0) | 1.00 | 0.00 | 0.00 | 0.00 | 1.00 | —   |
|                        | Small Tail Han | 383               |                  |      |     |     |                           |
|                        | Cele Black    | 68                |                  |      |     |     |                           |
|                        | Hu            | 83                |                  |      |     |     |                           |
|                        | Tan           | 22                |                  |      |     |     |                           |
|                        | Prairie Tibetan | 80              |                  |      |     |     |                           |
|                        | Suffolk       | 60                |                  |      |     |     |                           |
|                        | Sunite        | 70                |                  |      |     |     |                           |
| g.96101288G>A          |        | 0.99 (377)        | 0.01 (3)         | 0.00 (0) | 0.99 | 0.01 | 0.02 | 0.02 | 1.02 | 0.94 |
|                        | Small Tail Han | 380               |                  |      |     |     |                           |
|                        | Cele Black    | 68                |                  |      |     |     |                           |
|                        | Hu            | 82                |                  |      |     |     |                           |
|                        | Tan           | 23                |                  |      |     |     |                           |
|                        | Prairie Tibetan | 80              |                  |      |     |     |                           |
|                        | Suffolk       | 60                |                  |      |     |     |                           |
|                        | Sunite        | 70                |                  |      |     |     |                           |
| g.96092576G>A          |        | 0.68 (262)        | 0.3 (114)        | 0.02 (7) | 0.83 | 0.17 | 0.24 | 0.28 | 1.39 | 0.14 |
|                        | Small Tail Han | 383               |                  |      |     |     |                           |
|                        | Cele Black    | 68                |                  |      |     |     |                           |
|                        | Hu            | 83                |                  |      |     |     |                           |
|                        | Tan           | 23                |                  |      |     |     |                           |
|                        | Prairie Tibetan | 80              |                  |      |     |     |                           |
|                        | Suffolk       | 60                |                  |      |     |     |                           |
|                        | Sunite        | 70                |                  |      |     |     |                           |
| g.96101542G>A          |        | 0.65 (248)        | 0.32 (121)       | 0.03 (13) | 0.81 | 0.19 | 0.26 | 0.31 | 1.44 | 0.71 |
|                        | Small Tail Han | 382               |                  |      |     |     |                           |
|                        | Cele Black    | 68                |                  |      |     |     |                           |
|                        | Hu            | 83                |                  |      |     |     |                           |
|                        | Tan           | 23                |                  |      |     |     |                           |
|                        | Prairie Tibetan | 80              |                  |      |     |     |                           |
|                        | Suffolk       | 49                |                  |      |     |     |                           |
|                        | Sunite        | 70                |                  |      |     |     |                           |

Abbreviations: HE, heterozygosity; NE, number of effective alleles; PIC, polymorphism information content.
### TABLE 4 Polymorphism of CYP11A1 gene and population genetic analysis in seven sheep breeds

| Breed          | Number | Genotype frequency | Allele frequency | PIC | HE | NE | Chi-square test (p value) |
|----------------|--------|--------------------|------------------|-----|----|----|--------------------------|
|                |        | AA | AG | GG | A | G |                             |
| g.33222725A>G  | Small Tail Han | 383 | 0.97 (370) | 0.03 (13) | 0.00 (0) | 0.99 | 0.01 | 0.03 | 0.03 | 1.03 | 0.74 |
|                | Cele Black     | 68  | 0.96 (65) | 0.04 (3)  | 0.00 (0) | 0.98 | 0.02 | 0.04 | 0.04 | 1.04 | 0.85 |
|                | Hu            | 85  | 0.96 (82) | 0.04 (3)  | 0.00 (0) | 0.98 | 0.02 | 0.04 | 0.04 | 1.04 | 0.87 |
|                | Tan           | 23  | 0.87 (20) | 0.13 (3)  | 0.00 (0) | 0.94 | 0.06 | 0.11 | 0.12 | 1.14 | 0.74 |
|                | Prairie Tibetan | 80  | 0.81 (65) | 0.19 (15) | 0.00 (0) | 0.91 | 0.10 | 0.16 | 0.17 | 1.21 | 0.35 |
|                | Suffolk       | 60  | 0.97 (58) | 0.03 (2)  | 0.00 (0) | 0.99 | 0.02 | 0.03 | 0.03 | 1.03 | 0.90 |
|                | Sunite        | 70  | 0.90 (63) | 0.10 (7)  | 0.00 (0) | 0.95 | 0.05 | 0.09 | 0.10 | 1.10 | 0.66 |

Abbreviations: HE, heterozygosity; NE, number of effective alleles; PIC, polymorphism information content.

### TABLE 5 Association of different SNP genotypes of the STAR, HSD3B1 and CYP11A1 genes with litter size in Small Tail Han Sheep

| Gene       | Locus     | Genotype | First parity litter size (N) | Second parity litter size (N) | Third parity litter size (N) |
|------------|-----------|----------|-----------------------------|-------------------------------|-------------------------------|
|            |           |          |                             |                               |                               |
|            |           |          | 1.86 ± 0.03 N = 368         | 2.11 (220) ± 0.05             | 2.41 (85) ± 0.09              |
| STAR       | g.31959747T>C | TT       | 2.00 ± 0.27 (8)             | 2.33 (3) ± 0.33               | 3.50 (2) ± 0.50               |
|            |           | CC       | 1.50 (2) ± 0.50             | 1.00 (1) ± 0.00               | 0.00 (0) ± 0.00               |
|            |           |          |                             |                               |                               |
|            |           |          | 1.91 ± 0.06                 | 2.10 (88) ± 0.09              | 2.35 (31) ± 0.14              |
|            |           | GG       | 1.85 ± 0.05                 | 2.12 (117) ± 0.07             | 2.48 (48) ± 0.14              |
|            |           | GA       | 1.85 ± 0.10                 | 2.13 (24) ± 0.15              | 2.50 (10) ± 0.16              |
|            |           | AA       | 1.89 (289) ± 0.04           | 2.15 (176) ± 0.06             | 2.43 (67) ± 0.10              |
|            |           | CC       | 1.87 (380) ± 0.03           | 2.11 (226) ± 0.05             | 2.45 (87) ± 0.09              |
| HSD3B1     | g.96094239C>T | CT       | 2.50 (4) ± 0.29             | 2.67 (3) ± 0.33               | 2.00 (2) ± 1.00               |
|            |           | CC       | 1.89 (44) ± 0.10            | 2.30 (27) ± 0.13              | 2.46 (13) ± 0.27              |
|            |           | CT       | 1.89 (157) ± 0.05           | 2.08 (97) ± 0.08              | 2.59 (39) ± 0.14              |
|            |           | TT       | 1.85 (182) ± 0.05           | 2.09 (104) ± 0.08             | 2.27 (37) ± 0.12              |
|            |           | GG       | 1.87 (383) ± 0.03           | 2.11 (228) ± 0.05             | 2.44 (88) ± 0.09              |
|            |           | GA       | 1.87 (377) ± 0.03           | 2.11 (226) ± 0.05             | 2.43 (88) ± 0.09              |
|            |           | AA       | 1.67 (3) ± 0.33             | 1.00 (1) ± 0.00               | 0.00 (0) ± 0.00               |
|            |           | GG       | 1.87 (381) ± 0.03           | 2.11 (228) ± 0.05             | 2.43 (88) ± 0.09              |
|            |           | GA       | 1.87 (121) ± 0.06           | 2.06 (67) ± 0.09              | 2.15 (20) ± 0.15              |
|            |           | AA       | 1.69 (13) ± 0.17            | 1.91 (11) ± 0.25              | 2.00 (4) ± 0.41              |
| CYP11A1    | g.33222725A>G | AA       | 1.88 (370) ± 0.04          | 2.22 (218) ± 0.05             | 2.47 (87) ± 0.09             |
|            |           | AG       | 1.68 (13) ± 0.18           | 2.00 (10) ± 0.24              | 2.00 (4) ± 0.42              |
|            |           | CC       | 1.88 (377) ± 0.03          | 2.10 (227) ± 0.05             | 2.42 (89) ± 0.09              |
|            |           | CT       | 2.00 (7) ± 0.25            | 2.50 (2) ± 0.53              | 2.90 (2) ± 0.57              |

Note: Different letters for the groups indicate differences (p < 0.05).
However, the changes in the secondary structure of CYP11A1 protein were more complex, which may be one reason why the gene was significantly associated with litter size. However, the probability of two or three SNP mutations occurring at the same time is very low, so we analysed the effect of single SNP mutations on protein secondary structure. The results showed that STAR has few protein binding sites to begin with (Figure 3a), and after a single (Figure 3b,c) or multiple SNPs mutations (Figure 3d), there were still few binding sites. HSD3B1 originally had many binding sites (Figure 4a), but the degree of change caused by a single SNP mutation was low (Figure 4b–d). Only when three SNPs mutated simultaneously was the degree of change high (Figure 4e); however, the probability of this occurring is extremely low. In comparison, one SNP in the CYP11A1 gene brings significantly more changes to protein secondary structure (Figure 2e,f), which may explain why the g.33217408C>T SNP was associated with the litter size of sheep. And it might also be the reason why the SNPs in STAR and HSD3B1 gene were not associated with litter size, even though they also caused amino acid changes.

The 3-D structures before and after mutation in STAR, HSD3B1 and CYP11A1 were predicted via Phyre2 (Figure 5). The tertiary structure of CYP11A1 changed significantly after mutation (Figure 5e vs. Figure 5f), whereas STAR and HSD3B1 showed no significant changes. Due to the high similarity of the images, only one mutation result was presented for STAR and HSD3B1 in Figure 5. These 3-D computational models further confirm that the low degree of variation after single SNP mutation in the secondary structure of STAR and HSD3B1 would not likely affect protein function.

In addition, TMHMM analysis online (Krogh et al., 2001) predicted that the proteins encoded by STAR and CYP11A1 were outside the membrane before and after mutation. HSD3B1 had a predicted transmembrane region around amino acid 300 before and after the mutation. The results suggest that the mutations may not significantly affect the proteins’ transmembrane structures (Figure 6).
4 | DISCUSSION

Progestogens are important steroid hormones in human reproduction. The main tissues affected by progestogens include the uterus, vagina, cervix, breast, testicles and brain. The main effect of progesterone in the body is on the female and the male reproductive systems (Oettel & Mukhopadhyay, 2004). It is important to note that progestogens are often mistakenly confused with progesterone (P4). In fact, P4 is one of the most important progestogens. There are still a variety of different progestogens in the human body, but most of them are metabolites of P4 (Edwards et al., 2005) and are located downstream of P4 from the perspective of biosynthesis. P4 is produced from cholesterol with pregnenolone (P5) as a metabolic intermediate. In all mammals, the ovary is the primary site of P4 production, and the luteal cells of the ovary have key enzymes that convert cholesterol into P5 (via P4). The placenta accounts for the majority of P4 production in sheep, horse, and human (Malassine, 2001), while in other species, the corpus luteum is a major source of P4. Thus, P4 is the primary placental progestrone in sheep, horse and human.

As one of the most important progestogens, P4 is involved in the menstrual cycle, pregnancy and embryogenesis in human and other species (Taraborrelli, 2015). Recent research suggests that P4 affects reproduction (and cancer) through agonistic interactions with membrane P4 receptors (mPRs) (Thomas & Pang, 2012). However, more studies are needed to confirm this route (Valadez-Cosmes et al., 2016). It is well established that P4 transitions the endometrium into a secretory phase that prepares the uterus for implantation. P4 has an anti-mitogenic effect in endometrial epithelial cells, which reduces the oestrogenic effect (Patel et al., 2015), and seems to reduce the mother’s immune response during implantation and pregnancy, making pregnancy acceptable to the mother’s immune system (Di Renzo et al., 2016). Near the time of delivery, P4 reduces the contractile force of uterine smooth muscles (Wahabi et al., 2018), which helps prevent premature delivery (Di Renzo et al., 2016). According to Zakar and Hertelendy (2007), reducing P4 levels may facilitate onset of childbirth.

The ovarian steroidogenesis pathway includes STAR, HSD3B1, CYP1A1, CYP1B1, CYP11A1, CYP17A, and CYP19A1. STAR and HSD3B1 are related to the synthesis of sterol hormones (E2, P4 and androgen) that play a crucial role in the normal physiological effects of the ovary and maturation of the follicle (Guo et al., 2018). HSD3B1
STAR and CYP11A1 (P450scc) play important regulatory roles in steroid hormone biosynthesis.

STAR is expressed in the ovaries of a variety of animals, mainly overcoming the rate-limiting transfer of cholesterol from the outer mitochondrial membrane to P450scc (encoded by CYP11A1) located in the cell membrane (Yivgi-Ohana et al., 2009). Polytocous and monotonous Small Tail Han sheep had a similar level of STAR expression in ovary (Figure 1). However, STAR was highly expressed in the pituitary gland of monotonous sheep and low in the pituitary gland of polytocous sheep (Figure 1). Relevant studies have reported that ACTH (adrenocorticotropic hormone) produced by the anterior pituitary stimulates the adrenal cortex to synthesize glucocorticoids (Clark, 2016). The first step in glucocorticoid synthesis is the transfer of cholesterol to the mitochondrial matrix, which indirectly leads to a dramatic increase in the expression and function of steroid acute regulatory protein (STAR). This explains the high expression of STAR in the pituitary of monotonous sheep. However, whether the low expression of STAR in the pituitary is related to litter size has not been proved, and further experimental verification is still needed.

HSD3B1, the rate-limiting enzyme for sex hormone synthesis catalyses the initial step of steroid hormone production. Progesterone secretion by luteal cells requires P450scc and HSD3B1 enzyme activity (Chien et al., 2013). Studies have shown that the highest activity of HSD in the mitochondria of luteal cells may play an important role in the synthesis of progesterone (Chapman et al., 1992). This is consistent with the main expression of HSD3B1 gene in ovary observed in our study (Figure 1).

CYP19A1 and CYP1A1 in the ovarian steroidogenesis pathway are involved in the formation of ovarian steroids and may have important effects on the maintenance of luteal function and follicular dynamic development. CYP19A1 synthesizes oestrogen
from androgen the membrane of granulosa cells into (Miller & Auchus, 2011), thereby promoting follicular development. In vitro, oocytes with low expression of CYP1A1 (which encodes P450scc) had decreased rates of maturation (Pocar et al., 2004). P450scc is a mitochondrial enzyme that regulates the conversion of cholesterol into gestenolone (O'Hara et al., 2014). Also, studies on poultry have confirmed that the CYP11A1 gene has significant changes in expression during follicular development (Johnson & Woods, 2009), and CYP11A1 is regulated by hormones during follicular changes (Sechman et al., 2011).

To date, there have only been a few studies analysing SNPs in CYP11A1. A minor allele of CYP11A1, rs11638442, was proposed to increase endogenous levels of ouabain, an important factor in the cardiovascular system (Tripodi et al., 2009). Also, studies on poultry have confirmed that the CYP11A1 gene has significant changes in expression during follicular development (Johnson & Woods, 2009), and CYP11A1 is regulated by hormones during follicular changes (Sechman et al., 2011).

Related studies indicated that the SNP- derived mutations of CYP11A1 were closely related to reproduction and to the susceptibility to polycystic ovary syndrome, which can cause either chronic ovulation or anovulation (i.e., lack of ovulation) (Zhang et al., 2012). In the previous studies, the expression of the CYP11A1 gene in the ovarian tissue of monotocous Small Tail Han sheep was significantly higher than that of polytocous sheep (p < 0.01), suggesting that the CYP11A1 gene plays a negative regulation role during oestrus or ovulation (Tian et al., 2019). The CYP11A1 gene is also expressed in the hypothalamus and pituitary, but the specific role in these tissues still needs to be studied. In this study, the number of CYP11A1 mutation sites and amino acid changes was less than those of STAR and HSD3B1. However, according to bioinformatics analysis, the secondary structure change of CYP11A1 protein was more complex than that of STAR and HSD3B1 proteins, and there was a significant difference in litter size before and after mutation (p < 0.05). We speculate that g.33222725A>G and g.33217408C>T of CYP11A1 gene are the key influential mutation sites. Our study suggests that the CYP11A1 gene may be a polytocous gene; however, further investigation is required.
CONCLUSIONS

In general, the expression results showed that STAR gene was expressed in the hypothalamus-pituitary-ovary gonad axis, and there was a significant difference between polytocous sheep and monotocous sheep in the pituitary (p < 0.01). The HSD3B1 gene was mainly expressed in ovary, and there was significant difference between the ovary and other two tissues (p < 0.05). There was a significant difference in the association of litter size among the genotypes at g.33227257A>G and g.33217408C>T loci of CYP11A1 gene (p < 0.05). SNP g.33217408C>T of CYP11A1 caused an amino acid change at residue 41 (Ser to Phe). The physiological properties of Ser and Phe differ significantly, and this appeared to result in notable changes to the secondary and tertiary structures of CYP11A1. Our study provides a basis for further revealing the roles of the ovarian steroidogenesis pathway in sheep reproduction.

ACKNOWLEDGEMENTS

The authors are thankful to National Natural Science Foundation of China, grant number 31772580 and 31501941; Genetically Modified Organisms Breeding Major Program of China (2016ZX08009-003-006); Central Public-interest Scientific Institution Basal Research Fund, grant number 2018-YWF-YB-1 and Y2017JC24; the Earmarked Fund for China Agriculture Research System, grant number CARS-38; the Agricultural Science and Technology Innovation Program of China, grant number ASTIP-IAS13.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Wenping Hu: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Validation; Visualization; Writing-original draft; Writing-review & editing. Mingqiu Liu: Data curation; Formal analysis; Investigation; Methodology; Software; Validation; Visualization; Writing-original draft; Writing-review & editing. Zhilong Tian: Data curation; Investigation; Methodology; Validation. Qiuyue Liu: Funding acquisition; Resources. Zhuangbiao Zhang: Methodology; Software. Jishun Tang: Resources. Xiaoyun He: Resources. Yanyan Zhu: Resources. Yuanyuan Wang: Project administration; Supervision; Writing-review & editing. Ming-Xing Chu: Conceptualization; Funding acquisition; Project administration; Supervision; Writing-review & editing.

ETHICAL STATEMENT

All the experimental procedures mentioned in the present study were approved by the Science Research Department (in charge of animal welfare issues) of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (IAS-CAAS) (Beijing, China). Ethical approval on animal survival was given by the animal ethics committee of IAS-CAAS (No. IAS2020-63).

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms.485.

ORCID

Wen-Ping Hu https://orcid.org/0000-0002-8962-7541
Ming-Xing Chu https://orcid.org/0000-0002-5164-0310

REFERENCES

Abecia, J. A., Forcada, F., Palacin, I., Sanchez-Prieto, L., Sosa, C., Fernandez-Forens, A., & Meikle, A. (2015). Undernutrition affects embryo quality of superovulated ewes. Zygote, 23, 116–124.
Baker, M. E. (2013). What are the physiological estrogens?. Steroids, 78, 337–340.
Busada, J. T., & Cidlowski, J. A. (2017). Mechanisms of glucocorticoid action during development. Current Topics in Developmental Biology, 125, 147–170.
Chapman, J. C., Waterhouse, T. B., & Michael, S. D. (1992). Changes in mitochondrial and microsomal 3 beta-hydroxysteroid dehydrogenase activity in mouse ovary over the course of the estrous cycle. Biology of Reproduction, 47, 992–997.
Chien, Y., Cheng, W. C., Wu, M. R., Jiang, S. T., Shen, C. K., & Chung, B. C. (2013). Misregulated progesterone secretion and impaired pregnancy in Cyp11a1 transgenic mice. Biology of Reproduction, 89, 91.
Clark, B. J. (2016). ACTH action on STAR biology. Frontiers in Neuroscience, 10, 547.
Danielson, P. B. (2002). The cytochrome P450 superfamily: Biochemistry, evolution and drug metabolism in humans. Current Drug Metabolism, 3, 561–597.
Davis, G. H., Montgomery, G. W., Allison, A. J., Kelly, R. W., & Bray, A. R. (1982). Segregation of a major gene influencing fecundity in progeny of Booroola sheep. New Zealand Journal of Agricultural Research, 25, 525–529.
Di Renzo, G. C., Giardina, I., Clerici, G., Brillo, E., & Gerli, S. (2016). Progesterone in normal and pathological pregnancy. Hormone Molecular Biology and Clinical Investigation, 27, 35–48.
Edwards, H. E., Vimal, S., & Burnham, W. M. (2005). The acute anticonvulsant effects of deoxycorticosterone in developing rats: Role of metabolites and mineralocorticoid-receptor responses. Epilepsia, 46, 1888–1897. https://doi.org/10.1111/j.1528-1167.2005.00295.x
Frye, C. A. (2009). Steroids, reproductive endocrine function, and effect. A review. Minerva Ginecologica, 61, 541–562.
Fujita, T. (2010). Mineralocorticoid receptors, salt-sensitive hypertension, and metabolic syndrome. Hypertension, 55, 813–818. https://doi.org/10.1161/HYPERTENSIONAHA.109.149062
Gareis, N. C., Huber, E., Hein, G. J., Rodriguez, F. M., Salvetti, N. R., Angeli, E., Ortega, H. H., & Rey, F. (2018). Impaired insulin signaling pathways affect ovarian steroidogenesis in cows with COD. Animal Reproduction Science, 192, 298–312.
Gonzalez, F. J., & Gelboin, H. V. (1992). Human cytochromes P450: Evolution and cDNA-directed expression. Environmental Health Perspectives, 98, 81–85.
Greives, T. J., Mason, A. O., Scotti, M. A., Levine, J., Ketterson, E. D., Kriegsfeld, L. J., & Demas, G. E. (2007). Environmental control of kisspeptin: Implications for seasonal reproduction. Endocrinology, 148, 1158–1166. https://doi.org/10.1210/en.2006-1249
Guo, X., Wang, X., Di, R., Liu, Q., Hu, W., He, X., Yu, J., Zhang, X., Zhang, J., Broniowska, K., Chen, W., Wu, C., & Chu, M. (2018). Metabolic effects of FecB gene on follicular fluid and ovarian vein serum in sheep (Ovis aries). International Journal of Molecular Sciences, 19, 539.
Haas, D. M., & Ramsey, P. S. (2013). Progestogen for preventing miscarriage. Cochrane Database of Systematic Reviews, 10, CD003511.
Hanukoglu, I., Karavolas, H. J., & Goy, R. W. (1977). Progesterone metabolism in the pineal, brain stem, thalamus and corpus callosum of the female rat. *Brain Research*, 125, 313–324.

Johnson, A. L., & Woods, D. C. (2009). Dynamics of avian ovarian follicle development: Cellular mechanisms of granulosa cell differentiation. *General and Comparative Endocrinology*, 163, 12–17.

Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N., & Sternberg, M. J. E. (2015). The Phyre2 web portal for protein modeling, prediction and analysis. *Nature Protocols*, 10, 845–858.

Kohen, P., Castro, O., Palomino, A., Munoz, A., Christenson, L. K., Sierralta, W., Carvallo, P., Strauss, J. F. III, & Devoto, L. (2003). The steroidogenic response and corpus luteum expression of the steroidogenic acute regulatory protein after human chorionic gonadotropin administration at different times in the human luteal phase. *Journal of Clinical Endocrinology and Metabolism*, 88, 3421–3430. https://doi.org/10.1210/jc.2002-021916

Koritz, S. B. (1964). The conversion of pregnenolone to progesterone by small particles from rat adrenal. *Biochemistry*, 3, 1098–1102.

Krogh, A., Larsson, B., von Heijne, G., & Sonnhammer, E. L. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. *Journal of Molecular Biology*, 305, 567–580.

Malassine, A. (2001). Morphological variability and placental function. *Gynecologie, Obstetrique & Fertilite*, 29, 489–496.

McLaren, A. (1998). Genetics and human reproduction. *Trends in Genetics*, 14, 427–431.

Miller, W. L., & Auchus, R. J. (2011). The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocrine Reviews*, 32, 81–151.

National Committee on Livestock and Poultry Genetic Resources. (2012). *Genetic resources of livestock and poultry in China*. Sheep and goat. China Agriculture Press.

Nelson, L. R., & Bulun, S. E. (2001). Estrogen production and action. *Journal of the American Academy of Dermatology*, 45, S116–S124.

Notter, D. R. (2012). Genetic improvement of reproductive efficiency of sheep and goats. *Animal Reproduction Science*, 130, 147–151.

Oettel, M., & Mukhopadhyay, A. K. (2004). Progesterone: The forgotten hormone in men? *Aging Male*, 7, 236–257.

O’Hara, L., York, J. P., Zhang, P., & Smith, L. B. (2014). Targeting of GFP-Cre to the mouse Cyp11a1 locus both drives cre recombinase expression in steroidogenic cells and permits generation of Cyp11a1 knock out mice. *PLoS One*, 9, e84541. https://doi.org/10.1371/journal.pone.0084541

Pan, Z., Shi, S., Li, L., Wang, Z., Zhou, D., Li, K., Miao, B., Hu, W., Wang, X., Hu, X., Xu, Z., Wei, D., He, X., Yuan, L., Guo, X., Liang, B., Wang, R., Li, X., Cao, X., ... Li, Y. (2018). Whole-genome sequences of 89 Chinese sheep suggest role of RXFP2 in the development of unique horn phenotype as response to semi-feralization. *Gigascience*, 7, giy019. https://doi.org/10.1093/gigascience/giy019

Patel, B., Elguero, S., Thakore, S., Dahoud, W., Bedaivy, M., & Mesiano, S. (2015). Role of nuclear progesterone receptor isoforms in uterine pathophysiology. *Human Reproduction Update*, 21, 155–173.

Pocar, P., Augustin, R., & Fischer, B. (2004). Constitutive expression of CYP1A1 in bovine cumulus oocyte-complexes in vitro: Mechanisms and biological implications. *Endocrinology*, 145, 1594–1601.

Sechman, A., Pawlowska, K., & Hrabia, A. (2011). Effect of 3,3′-5-triiodothyronine and 3,5-diiodothyronine on progesterone production, cAMP synthesis, and mRNA expression of STAR, CYP11A1, and HSD3B genes in granulosa layer of chicken preovulatory follicles. *Domestic Animal Endocrinology*, 41, 137–149. https://doi.org/10.1016/j.domaniend.2011.05.007

Sun, M., Yang, X., Ye, C., Xu, W., Yao, G., Chen, J., & Li, M. (2012). Risk-association of CYP11A1 polymorphisms and breast cancer among Han Chinese women in Southern China. *International Journal of Molecular Sciences*, 13, 4896–4905.

Tajima, K., Dantes, A., Yao, Z., Sorokina, K., Kotsui, F., Seger, R., & Amsterdam, A. (2003). Down-regulation of steroidogenic response to gonadotropins in human and rat preovulatory granulosa cells involves mitogen-activated protein kinase activation and modulation of DAX-1 and steroidogenic factor-1. *Journal of Clinical Endocrinology and Metabolism*, 88, 2288–2299. https://doi.org/10.1210/jc.2002-020913

Tang, J., Hu, W., Chen, S., Di, R., Liu, Q., Wang, X., He, X., Gao, S., Zhang, X., Zhang, J., Chen, W., & Chu, M. (2019). The genetic mechanism of high prolificacy in small tail han sheep by comparative proteomics of ovaries in the follicular and luteal stages. *Journal of Proteomics*, 204, 103394.

Taraborelli, S. (2015). Physiology, production and action of progesterone. *Acta Obstetricia Et Gynecologica Scandinavica*, 94(Suppl 161), 8–16.

Thomas, P., & Pang, Y. (2012). Membrane progesterone receptors: Evidence for neuroprotective, neurosteroid signaling and neuroendocrine functions in neuronal cells. *Neuroendocrinology*, 96, 162–171. https://doi.org/10.1159/000339822

Tian, Z., Liu, Q., Wang, X., Di, R., Hu, W., Wang, Y., Zhang, X., Zhang, J., & Chu, M. (2019). Expression of CYP11A1, CYP17A1 and CYP19A1 genes in hypothalamus-pituitary-ovary axis of Small Tail Han Sheep. *Journal of China Agricultural University*, 204, 87–93.

Tripodi, G., Citterio, L., Kounznetsova, T., Lanzani, C., Florio, M., Modica, R., Messaggio, E., Hamlyn, J. M., Zagato, L., Bianchi, G., Stassen, J. A., & Manunta, P. (2009). Steroid biosynthesis and renal excretion in human essential hypertension: Association with blood pressure and endogenous ouabain. *American Journal of Hypertension*, 22, 357–363. https://doi.org/10.1038/ajh.2009.3

Valadz-Cosmes, P., Vazquez-Martinez, E. R., Cerbon, M., & Camacho-Arroyo, I. (2016). Membrane progesterone receptors in reproduction and cancer. *Molecular and Cellular Endocrinology*, 434, 166–175.

Vallee, M. (2016). Neurosteroids and potential therapeutic: Focus on pregenolone. *Journal of Steroid Biochemistry and Molecular Biology*, 160, 78–87.

Wahabi, H. A., Fayed, A. A., Esmaeil, S. A., & Bahkali, K. H. (2018). Progestogen for treating threatened miscarriage. *Cochrane Database Systematic Review*, 8, CD005943.

Yi-vi-Ohana, N., Sher, N., Melamed-Book, N., Eimerl, S., Koler, M., Manna, P. R., Stocco, D. M., & Orly, J. (2009). Transcription of steroidogenic acute regulatory protein in the rodent ovary and placenta: Alternative modes of cyclic adenosine 3’,5’-monophosphate dependent and independent regulation. *Endocrinology*, 150, 977–989. https://doi.org/10.1210/en.2008-0541

Zakar, T., & Hertelendy, F. (2007). Progesterone withdrawal: Key to parturition. *American Journal of Obstetrics and Gynecology*, 196, 289–296.

Zhang, C. W., Zhang, X. L., Xia, Y. J., Cao, Y. X., Wang, W. J., Xu, P., Che, Y. N., Wu, X. K., Yi, L., Gao, Q., & Wang, Y. (2012). Association between polymorphisms of the CYP11A1 gene and polycystic ovary syndrome in Chinese women. *Molecular Biology Reports*, 39, 8379–8385.

Zhang, Z., Liu, Q., Di, R., Hu, W., Wang, X., He, X., Ma, L., & Chu, M. (2019). Single nucleotide polymorphisms in BMP2 and BMP7 and the association with litter size in Small Tail Han sheep. *Animal Reproduction Science*, 204, 183–192.

Zheng, J., Wang, Z., Yang, H., Yao, X., Yang, P., Ren, C., Wang, F., & Zhang, Y. (2019). Pituitary transcriptomic study reveals the differential regulation of IncRNAs and mRNAs related to prolificacy in different FecB genotyping sheep. *Genes*, 10(2), 157.