Association of VEGF and IGF2 Polymorphisms with Lambing Number of Small Tail Han Sheep

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ABSTRACT

This study aimed to clarify the relationship between VEGF and IGF2 single nucleotide polymorphisms (SNP) and lambing number in small tail Han sheep, and to provide the basis for molecular marker-assisted selection (MAS) of sheep fecundity. A total of 519 small tail Han sheep was selected in this study, and PCR-RFLP and PCR-SSCP were performed to detect the polymorphism of VEGF and IGF2, and also analyzed the relationship between the SNPs and lambing number of small tail Han sheep. Two SNPs were identified in small tail Han sheep: g. 14752 C>T, a C→T change at 14752 bp mutation in the fifth intron of VEGF; and g. 165 G>A, a G→A change at 165 bp mutation in the first expressed region of IGF2. Three genotypes CC, CT and TT were detected in the VEGF g. 14752 C>T SNP, and the association result showed that the lambing number of TT type in the small tail Han sheep was higher than that in CT and CC genotypes for 0.7 (P < 0.05) and 1.05 (P < 0.05), respectively. The lambing number of sheep in CT genotype was 0.35 more than that in CC genotype, however, there was no significant difference among lambing number in CC, CT and TT of small tail Han sheep (P > 0.05). The GG, GA and AA genotypes were detected in the IGF2 g. 165 G>A mutation in small tail Han sheep, and genotype AA had a single nucleotide mutation in g. 165 G>A mutation in small tail Han sheep, however there was no significant difference among lambing number in GG, GA and AA genotypes in lambing number. Together, our results indicated that the SNP VEGF g. 14752 C>T might be a referential significance for the breeding of lambing number of small tail Han sheep, and while the IGF2 g. 165 G>A was not suitable.

INTRODUCTION

The quantitative trait with important economic value is litter size of farm animals. Due to the sex restriction, age and low heritability, traditional selection was difficult to improve the litter size of sheep (He et al., 2019). However, if the QTLs of genetic markers closely associated with lambing numbers could be found, improvements would be made in selection and efficiency for low genetic traits. Vascular endothelial growth factor (VEGF), a member of VEGF family, is the main factor for promoting angiogenesis. Of which, there are seven members have been found including VEGF-A/B/C/D/E/F and placental growth factor (PLGF) (Shibuya, 2013). VEGF is located on human chromosome 6p12-21, and is approximately 1.6kb in length and contains 8 exons and 7 introns (Brioude et al., 2013). It has various alternatives splicing in human, which can encode proteins of 121, 145, 165, 189 and 206 amino acid, and each has different biological activities (Roskoski, 2007). Five isoforms of VEGF were all dimer glycoprotein, which widely distributed in heart, lung, lymph, thyroid gland, skeletal muscle, central nervous system and other tissues (De Bock et al., 2013; Potente et al., 2011). When combined with its receptor, VEGF can promote the hyperplasia of vascular endothelial cell and improve vascular permeability (Mac Gabhann et al., 2008). It is also critical for the normal development and maintaining of follicle and lithium, periodical change of the...
endometrium, attached implant of embryonic development and other functions in female animals (Malyasz-Cymborska et al., 2014). In addition, VEGF also plays an essential role in blastocyst implantation and placentation development. Especially, the expression level of VEGF isoform of 165 amino acid and alkaline fibroblast bFGF were related to the weight of placenta in the whole gestation (Keshavarzi et al., 2019). In a study by Xinrong Wang et al. on small-tailed Han sheep, lake sheep, Aohan fine wool sheep and Tibetan sheep in different environments and lambing numbers in China, it was found that ovarian vascular diameter was significantly larger in high-fertility breeds than in low-fertility breeds, and the VEGF gene transcript and protein expression were significantly and positively correlated with lambing numbers (P<0.05) (Wang et al., 2020). The angiogenesis-related factor VEGF is essential for the ovariuly cycle of females, including follicle development, ovulation and luteal formation (Kona et al., 2021; Wang et al., 2020). The study shows that have shown that expression of the VEGF gene stimulates angiogenesis and follicle development (Lupicka et al., 2019).

IGF2 is a peptide hormone that participates in the IGF axis, which plays an important role in the promotion of cell proliferation and the differentiation of preimplantation embryos (Nordin et al., 2014). The previous study showed that insulin-like growth factors (IGFs) were involved in follicular development and steroidogenesis in the ovary, the proliferation and differentiation of the uterine endometrium, and the implantation of the embryo (Hsu et al., 2019). The expression of IGF2 was higher in ovarian venous effluents than that in the peripheral circulation, which implied that this peptide sourced the ovarian (Younis et al., 2020). The concentrations of IGF2 are significantly higher in ovarian venous effluents than in the peripheral circulation, implying an ovarian source for this peptide. Some authors have demonstrated a direct participation of IGF2 in the reproductive function in mouse and farm animals (Badinga et al., 1999). Many previous studies showed that SNPs of IGF2 are related to growth traits in swine or milk production traits in cattle (Jungerius et al., 2004; Simonetti et al., 2018; Bagnicka et al., 2010; Berkowicz et al., 2011). But there are also a few researches about the association between reproductive traits and the SNPs within IGF2. Rempel et al. (2010) reported that four IGF2 SNPs were associated with age at puberty in swine, and it have additive or dominant effects (3.2 to 5.8 d; P ≤ 0.0052), and the SNPs within IGF2 (A=0.26 piglets; P=0.0032) were also associated with number of piglets born dead (Rempel et al., 2010).

In recent years, reports of VEGF and IGF2 gene interactions have been increasing. In studies on rhesus monkeys, VEGF and IGF2 were found to exert an important influence on the function of early blastoderm trophoderm cells, and here it was observed that the expression of IGF2 in trophoderm cells was reduced following the addition of anti-VEGF growth factor antibodies, affecting the regulation of VEGF-IGF2-MMPs and thus blastoderm formation was hindered (Ghosh et al., 2011). In an exploration of individual development of the mouse imprinted gene IGF2/H19, it was found that increased IGF2 mRNA transcription significantly affected VEGF expression, suggesting that IGF2 interacts with VEGF during mammalian growth and development (Kawahara et al., 2010).

The use of molecular information in sheep breeding programs may enhance genetic gains by increasing the accuracy of genetic evaluation and decreasing generation intervals (Stinckens et al., 2010). There are a number of sheep breeds in China, of which show superior performance. small tail Han sheep that has significant characteristics of high proficiency and year round estrus is an excellent local sheep breed in China. Now, few research results in the nucleotide sequence and variation of the IGF2 in sheep have been reported. Small tail Han sheep was selected in this research which used for analyzing the relationship between lambing number and polymorphisms of VEGF and IGF2. In order to find the genetic markers related to lambing number, and provide a scientific basis for MAS of prolific sheep breeds.

**MATERIALS AND METHODS**

**Preparation of simples and DNA extraction**

All experiments involving animals were authorized by the Animal Ethics Committee of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (No. IAS2020-63).

Blood samples were collected from 519 female small tail Han sheep for DNA extraction, and that divided two groups 246 sheep used to PCR-RFLP and 273 sheep for PCR-SSCP detection, respectively. These ewes were randomly selected in Jiaxiang Sheep Breeding Farm, Shandong Province, P.R. China. No selection on lambing number or other fertility traits was carried out in the flock over past years. Acid citrate glucose was used as the anticoagulant.

Genomic DNA (Tiangen, Beijing, P.R. China) was extracted from whole blood according to the phenol-chloroform method, then dissolved them in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) and stored at -20 ℃.

Primer P3 was designed according to the sequence of Ovis aries VEGF gene (GenBank No. NC_007324), the other four primers were adopted from the published
article (Jin et al., 2010). All primers were used to amplify the region of exon 2 to exon 6 and partial introns of sheep VEGF gene. The primers were synthesized by Beijing Tianyihuiyuan Biotechnology Co. Ltd. (Beijing, P.R. China). Information of primers was listed in Table I.

PCR reactions of VEGF were performed in 20 µL volume, containing 10×PCR buffer (containing Mg²⁺) 2 µL, 1.5 µL of 2.5 mmol/L dNTPs, 1.0 µL of 10 µmol/L each primer, 3.0 µL of 50 ng/µL genomic DNA, 0.5 µL of 2.5 U/µL Taq DNA polymerase (Promega, Madison, WI, USA), and the rest was ddH₂O. PCR conditions were as follows: initial denaturation at 95 ℃ for 5 min, followed by 35 cycles of denaturation at 95 ℃ for 30 s, annealing at 59 ℃ for 30 s, extension at 72 ℃ for 30 s, with a final extension at 72 ℃ for 10 min, then kept at 4 ℃ (Eppendorf AG, Hamburg, Germany).

AvaI, PstI and BsaHI were selected using in the enzyme reaction of P5 PCR products. Enzyme digestion reaction was performed in 10 µL volume, containing PCR amplification product 4.0 µL, restriction enzyme 0.25 µL, 10×buffer 1.0 µL, and the rest was ddH₂O. AvaI, PstI and BsaHI were all digested at 37 ℃ overnight, the enzyme-digested products were detected by electrophoresis on 12% polyacrylamide gel (29:1), 150 V for 5 h, and then silver nitrate staining was used to identify the bands, then photographed and analyzed using an AlphaImager™ 2200 and 1220 Documentation and Analysis Systems (Alpha Innotech Corporation, San Leandro, CA, USA).

PCR amplification and PCR-SSCP analysis
Five pairs of primers were designed according to mRNA sequence of sheep IGF2 gene (GenBank accession number NM_001009311) and DNA sequence of cattle IGF2 gene (GenBank accession number NC_007330). The primers were synthesized by Shanghai Invitrogen Biotechnology Co. Ltd. (Shanghai, China). Primer sequence, expected size were listed in Table I.

Table I. Primers of sheep VEGF and IGF2.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Primer sequence (5'-3')</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>P1</td>
<td>F: 5'-CTGCCGCTGCCCCATCTT-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-CCAACAGACCTTCCCACATC-3'</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>F: 5'-CCTTTTCCCTGTTGGTATAC-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-CACCTGCATTTCTGTTTGATC-3'</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>F: 5'-CTGCGCATCTTGGTTGAGG-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-CCGGTAAACCAACTCTGAGC-3'</td>
<td>308</td>
</tr>
<tr>
<td></td>
<td>P4</td>
<td>F: 5'-TCTGTCTCCTCCGGTGTG-3'</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>R: 5'-CTCTGACCTTTGCTGTCGCT-3'</td>
<td>327</td>
</tr>
<tr>
<td></td>
<td>P5</td>
<td>F: 5'-GGGCTATGCAATCCAGTCTT-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-CCGGCTATGGTGATGTCGTG-3'</td>
<td>238</td>
</tr>
<tr>
<td>IGF2</td>
<td>P1</td>
<td>F: GAGGGGACGAAGAGGATCTATGTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CAGTTTCGAGCGGAGGTTGGGATT</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>F: ATGGGGATCACAGCAGGAAAGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AGAAGCCGGGCGTCCACACAGC</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>F: AGGCGTGCCATCTGGGAAGAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CACGCTGCTAGAGGACAGCAC</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td>P4</td>
<td>F: CCCGTTGGCAAGGTCTTCCCAAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: ATCGCTGATACGCTTGGGAAGAG</td>
<td>213</td>
</tr>
<tr>
<td></td>
<td>P5</td>
<td>F: AAGTGAGCCAAAAAGTGCTGTATAT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GCTGATGGGAGGTTATGATT</td>
<td>321</td>
</tr>
</tbody>
</table>
Table II. Allele and genotype frequencies of PCR amplification in small tail Han sheep.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus Number</th>
<th>Number</th>
<th>Genotype frequency</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>g. 14752 C&gt;T</td>
<td>244</td>
<td>CT 0.524(128)</td>
<td>C 0.524</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TT 0.406(99)</td>
<td>T 0.406</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>g. 14758 C&gt;T</td>
<td>241</td>
<td>GT 0.876(211)</td>
<td>G 0.876</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T 0.124</td>
</tr>
<tr>
<td></td>
<td>g. 14908 C&gt;T</td>
<td>246</td>
<td>CT 0.524(128)</td>
<td>C 0.524</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TT 0.455(112)</td>
<td>T 0.455</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF2</td>
<td>g. 165G&gt;A</td>
<td>273</td>
<td>GA 0.432(118)</td>
<td>G 0.432</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AA 0.410(112)</td>
<td>A 0.410</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The numbers in the brackets are the genotype individuals.

Statistical analysis

Association of different genotypes with lambing number in small tail Han ewes was analyzed using the following model:

\[ y_{ijklm} = \mu + S_i + LS_j + P_k + Gl + e_{ijklm} \]

Where \( y_{ijklm} \) is phenotypic value of litter size; \( \mu \) is population mean; \( S_i \) is the fixed effect of the \( i^{th} \) ram; \( LS_j \) is the fixed effect of the \( j^{th} \) lambing season (\( j = 1, 2, 3, 4 \)); \( P_k \) is the fixed effect of the \( k^{th} \) parity (\( k = 1, 2, 3 \)); \( G_l \) is the fixed effect of the \( l^{th} \) genotype (\( l = 1, 2, 3 \)); and \( e_{ijklm} \) is the random residual. Analysis was performed using the general linear model procedure and least significant difference (LSD) of SPSS (V17.0). Mean separation procedures were conducted using a least significant difference test.

RESULTS

**VEGF and IGF2 amplification**

Genomic DNA of small tail Han sheep was amplified using primers for VEGF and IGF2. PCR products were detected by running a 2% agarose gel electrophoresis. The amplified products were consistent with the target ones and had a good specificity, which could be directly used for subsequent study, including cloning, sequencing and sequence comparative analysis.

**VEGF and IGF2 polymorphism**

One enzyme digestion site AvaI was detected in the 238 bp fragment amplified by primer P5, which produced two bands with sizes of 39 and 199 bp by enzyme digestion. Three genotypes [CC (39/199 bp), CT (39/199/238 bp) and TT (238 bp)] were detected in small tail Han sheep (Fig. 1).

Two enzyme digestion sites PstI were detected in the fragment amplified by primer P5, which produced three bands with sizes of 16, 49 and 173 bp by enzyme digestion. Two genotypes [GG (16/49/173 bp) and GT (16/49/65/173 bp)] were detected in small tail Han sheep (Fig. 2).

Fig. 1. AvaI-RFLP analysis on amplified region by primer P5 of VEGF in sheep.
Lanes 1 to 3: genotype CC; lanes 4 to 6: genotype TT; lanes 7 to 9: genotype CT; M: DNA Marker I (TIANGEN, China).

Fig. 2. PstI-RFLP analysis on amplified region by primer P5 of VEGF in sheep.
Lanes 1 to 4: genotype GT; lanes 5 to 8: genotype GG; M: 20 bp DNA Ladder Marker (TAKARA).
One enzyme digestion site BsaHI was detected in the fragment amplified by primer P5, which produced two bands with sizes of 42 and 196 bp by enzyme digestion. Three genotypes [CC (42/196 bp), CT (42/196/238 bp) and TT (238 bp)] were detected in small tail Han sheep (Fig. 3).

No polymorphism was detected in products amplified by primers P1 to P4. The PCR-SSCP products of IGF2 only the PCR products amplified by primer P1 displayed polymorphism. Three genotypes (GG, GA and AA) were detected in small tail Han sheep (Fig. 4).

Sequencing analysis of PCR amplified fragments

In the sequence amplified by VEGF primers, it was found that only the PCR product amplified by primer P5 was polymorphic twenty PCR-RFLP products of each primer were selected at random and used for cloning and sequencing. The result showed that three polymorphic sites were detected in the products amplified by primer P5: a C→T change at 14752 bp (relative to NC_007324, and the same below) and a G→T change at 14758 bp in the fifth intron, a C→T change at 14908 bp in the sixth exon (Figs. 5-7), while no polymorphisms was detected in products amplified by primers P1 to P4.
It was found in the sequence amplified by the primer of IGF2: the PCR products amplified by primer P1 displayed polymorphism. Three genotypes (GG, GA and AA) were detected in small tail Han sheep. For primer P1, sequencing revealed one nucleotide mutation (G165A) (Fig. 8) of IGF2 between GG and AA genotypes. This mutation located in the 5' UTR of IGF2 in sheep. Fig. 8. Sequence comparison of 165 bp of GG and AA genotypes of IGF2 primer P1 in sheep. Genotype and allele frequencies of VEGF and IGF2 in small tail Han sheep

Allele and genotype frequencies of VEGF and IGF2 in small tail Han sheep breeds are shown in Table II. Association of different VEGF and IGF2 genotypes with lambing number in small tail Han sheep

Least squares mean and standard error for lambing number of different genotypes at three loci of VEGF in small tail Han sheep are shown in Table III.

Table III. Least squares mean and standard error for lambing number of different genotypes at three loci of VEGF in small tail Han sheep.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Genotype</th>
<th>Number</th>
<th>Lambing number</th>
</tr>
</thead>
<tbody>
<tr>
<td>g. 14752 C&gt;T</td>
<td>CC</td>
<td>128</td>
<td>1.81±0.11 *</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>99</td>
<td>2.16±0.14 b</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>17</td>
<td>2.86±0.20 a</td>
</tr>
<tr>
<td>g. 165G&gt;A</td>
<td>GG</td>
<td>118</td>
<td>1.45±0.12 c</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>112</td>
<td>1.89±0.14 a</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>43</td>
<td>2.57±0.17 a</td>
</tr>
</tbody>
</table>

DISCUSSION

Association between the SNP of VEGF and reproductive performance

VEGF is one of the most important factors in your angiogenesis. It is widely present in various organs of humans and other animals and is able to participate in all stages of follicular development (Li et al., 2020). This study indicated that as for the VEGF g.14752C>T in the fifth intron, small tail Han sheep ewes with genotype TT had 0.7 (P<0.05) and 1.05 (P<0.05) lambing number more than that with CT or CC, respectively. The ewes with genotype CT had 0.35 lambs more than those with CC (P<0.05). This indicates that VEGF is able to influence the number of lambs produced in small tail Han sheep, which is consistent with numerous reports. Studies showed that the concentration of VEGF in placenta increased gradually with the sustaining of gestation, while in RSA patients, the concentration of VEGF receptor decreased gradually in the first three months of gestation (Olaya et al., 2019). Further studies have confirmed that VEGF plays a central role in the development of the uterus and ovary during ovarian development, because the immunoreactivity of VEGF and IL-1 in the uterus and ovary in the ischemia-reperfusion group is higher than that in the control group (Ersoy-Canillioglu et al., 2020). The vascularization induced by VEGF was crucial to the determination of advantage follicles and the delivery of gonadotropic hormone in follicle development (Babitha et al., 2014). VEGF overexpression resulted in a significant increase in embryonic growth rate, while the interference with VEGF
expression resulted in poor embryo quality and affected embryo development, which leads to poor embryo quality and consequently low fertility (Carr et al., 2014). Zhang et al. (2019) showed that the regulation of NF-kB signaling promotes the promotion of ovarian development (Zhang et al., 2019).

**Association between SNP of IGF2 and reproductive performance**

IGF2 is one of the earliest endogenous imprinted genes discovered (Brioude, 2021). IGF2 promoter activity is tissue-specific and related to developmental stage in an important link in human development. IGF2 is a major promoter of embryonic development, generally speaking, it mainly exists in the present of the fetus under normal physiological conditions. In this study, one nucleotide mutation (g. 165 G>A) of IGF2 was identified and the mutation located in the 5’ UTR of sheep IGF2. It is an excellent deal of evidence that IGF1 and IGF2 play autocrine and paracrine roles in the regulation of ovarian development. The findings suggest that the lack of IGF2-AS in villi is related to humans (Wu et al., 2020). Stinckens et al. showed that sow reproductive performance may be related to imprinted markers of their sire’s IGF2 and that IGF2 expression differs in the ovarian follicles of sows (Stinckens et al., 2010). Different molecular forms of IGFFBPs regulate reproductive function as IGFs by increasing gene expression and enzyme activity to regulate steroids, thus regulating ovarian development (Higuchi et al., 2020). Tkachenko et al found that IGF2 addition increased follicle survival and affected granulosa cell proliferation. These data suggested that IGF2 produced by sinus follicles is responsive to steroid hormone regulation and can act as a paracrine factor that positively affects antral follicle development and function in primates (Tkachenko et al., 2021). Got through the gene expression profile by microarray gene analysis, and verified the key genes by GCs qPCR. The increase was accompanied by the expression levels of IGF2, IGF receptor and IGF activated genes of follicular size, and reached the peak of IGF2 before ovulation (Botkjaer et al., 2019). Hsu et al. (2019) in mouse studies that there are a large number of IGF axons, including IGF2, IGF receptor and IGF activating genes in the process of ovarian ovulation, which proves that IGF2, IGF receptor and IGF activating genes regulate tissue regeneration after ovulation (Hsu et al., 2019). IGF2 and IGF1R mRNAs were found to be present in human spermatozoa and their transcription levels were positively correlated with sperm concentration and total sperm count (Cannarella et al., 2020). Studies have found that IGF2 methylation may lead to down-regulation of chronic villi and affect normal pregnancy (Wu et al., 2020). IGF2 contributes to the development of preimplantation blastocysts (Park et al., 2011), trophoblast invasion (Hiden et al., 2009), and decidualization (Suzukawa et al., 2020).

**CONCLUSION**

The results preliminarily indicated that allele g.14752 C>T in the fifth intron of VEGF is an effective potential marker which can improve lambing number in sheep and the SNP (g. 165 G>A) within IGF2 may have no effect on lambing number in sheep. VEGF is important for regulating the ovulatory cycle in females, including the processes of follicle development, ovulation and corpus luteum formation. Many studies have shown that VEGF genes can stimulate angiogenesis and follicle development (Chen et al., 2015; Carret et al., 2016). It has been shown that the expression and protein diversity of VEGF genes in the vasculature increases with increasing lambing numbers. In an RNA-seq analysis of VEGF expression patterns in the ovaries of Hetian and Cele sheep, it was shown that VEGF expression had a significant effect on reproductive efficiency (Chen et al., 2015). This suggests that VEGF could be one of the key factors influencing reproductive performance in sheep.

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**IRB approval**

This study and all the experimental procedures were approved by the Science Research Department (in charge of animal welfare issues), Institute of Animal Science, Chinese Academy of Agricultural Sciences (IAS-CAAS) (Beijing, China).

**Ethical statement**

Ethical approval was also provided by the animal ethics committee of IAS-CAAS (No. IAS2021-25).
Statement of conflict of interest
The authors have declared no conflict of interest.

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