Pakistan J. Zool., vol. 57(3), pp 1197-1206, 2025 DOI: https://dx.doi.org/10.17582/journal.pjz/20211002111008

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

Peng Wang[1,2], Cai Xia Geng[3], Bao Yun Zhang[4], Hong Quan Chen[3], Ping Qing Wang[4], Yu Fang Liu[2]* and Ming Xing Chu[1]*

[1]*Key Laboratory of Animal Genetics, Breeding and Reproduction of Ministry of Agriculture and Rural Affairs, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100193, China*
[2]*College of Life Sciences and Food Engineering, Hebei University of Engineering, Handan 056021, China*
[3]*College of Animal Science and Technology, Anhui Agricultural University, Hefei 230036, China*
[4]*Bioengineering College, Chongqing University, Chongqing 400030, China*

Article Information
Received 02 October 2021
Revised 15 September 2022
Accepted 12 October 2022
Available online 30 January 2024 (early access)
Published 01 May 2025

Authors' Contribution
MC and YL designed the study. PW, CG and HC conducted the experiments. PW, BZ and HC analyzed the data and drafted the manuscript. CG, BZ, HC, PW, YL and MC helped in preparation of the manuscript.

Key words
Sheep, Prolificacy, *VEGF, IGF2*, Lambing number

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 the 5' regulatory region of the *IGF2* gene g. 165 G>A compared to genotype GG. No significant differences (P >0.05) were found between the 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, 2021). 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

* Corresponding author: mxchu@263.net, aigaiy@126.com
0030-9923/2025/0003-1197 $ 9.00/00

endometrium, attached implant of embryonic development and other functions in female animals (Malysz-Cymborska *et al*., 2014). In addition, *VEGF* also plays an essential role in blastocyst implantation and placental 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 ovulatory 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 (*IGF*s) 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; $P \leq 0.0052$), 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 trophectoderm cells, and here it was observed that the expression of *IGF2* in trophectoderm 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°C.

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 Mg^{2+}) 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 ddH_2O. PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s, extension at 72°C for 30 s, with a final extension at 72°C for 10 min, then kept at 4°C (Eppendorf AG, Hamburg, Germany).

*Ava*I, *Pst*I and *Bsa*HI 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 ddH_2O. *Ava*I, *Pst*I and *Bsa*HI were all digested at 37°C 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.

The mixture for PCR-SSCP of *IGF2* gene was enzyme digestion reaction was performed in 10 μL volume, PCR amplification product 3.0 mL, other 7 μL solution is 98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 10 mmol/L EDTA (pH 8.0), 10% glycerol was transferred in an Eppendorf tube, denatured at 98°C for 10 min, then cooled down at -20°C for 10 min (Eppendorf AG, Hamburg, Germany). Secondly, they were separated by an electrophoresis on a 12% neutral polyacrylamide gel (acrylamide: bisacry lamide= 39:1) at 9-15 V/cm for 14-16 h at 4°C, then stained with silver nitrate (silver staining). Finally, patterns and analysis were achieved using AlphaImager™ 2200 and 1220 Documentation and Analysis Systems (Alpha Innotech Corporation, San Leandro, CA, USA).

Table I. Primers of sheep *VEGF* and *IGF2*.

Gene	Primer name	Primer sequence (5'-3')	Product size (bp)
VEGF	P1	F: 5'-CTGCCGCTGCCCATTCTT-3'	84
		R: 5'-CCAACAGACCTTCCCACTCATC-3'	
	P2	F: 5'-CCTTTCCCGTGGTGGTTAC-3'	320
		R: 5'-CACCTGCATGGTGATGTTGA-3'	
	P3	F: 5'-CTGCCTAGCATTGTTACAAGG-3'	308
		R: 5'-CCGTGAAACCAACTCTCAGAC-3'	
	P4	F: 5'-TCTTGTCTTCCGCTGTGGCAT-3'	327
		R: 5'-CTCTGACTTGCTCGCCCTCTG-3'	
	P5	F: 5'-TGGAGGCTAGGACTGTGCTTT-3'	238
		R: 5'-GCGGCTATGGGTAGTTCTGTG-3'	
IGF2	P1	F: GAGGGGACGAAGAGTCACTGTT	198
		R: CAGTTCTGAGCAGGTGGGGATT	
	P2	F: ATGGGGATCACAGCAGGAAAGT	151
		R: AGAAGCCGCGGTCCCCACAGAC	
	P3	F: AGCCGTGGCATCGTGGAAGAG	141
		R: CACGGTCGTAGAGGCAGACAC	
	P4	F: CCCGTGGGCAAGTTCTTCCAAT	213
		R: ATCGCTGGATGCCTCGGAAGAG	
	P5	F: AAGTGAGCCAAAGTGTCGTAAT	321
		R: GCTGATTGAGGGGTTTATGATT	

Table II. Allele and genotype frequencies of PCR amplification in small tail Han sheep.

Gene	Locus	Number	Genotype frequency			Allele frequency	
VEGF	g. 14752 C>T	244	CC	CT	TT	C	T
			0.524(128)	0.406(99)	0.070(17)	0.727	0.273
	g. *14758* C>T	241	GG	GT		G	T
			0.876(211)	0.124(30)		0.938	0.062
	g. *14908* C>T	246	CC	CT	TT	C	T
			0.098(24)	0.455(112)	0.447(110)	0.325	0.675
			0.524(128)	0.406(99)	0.070(17)	0.727	0.675
IGF2	g. 165G>A	273	GG	GA	AA	G	A
			0.432(118)	0.410(112)	0.158(43)	0.637	0.363

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:

$$y_{ijklm}=\mu+S_i+LS_j+P_k+G_l+e_{ijklm}$$

Where y_{ijklm} is phenotypic value of litter size; μ 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 *Ava*I 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 *Pst*I 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).

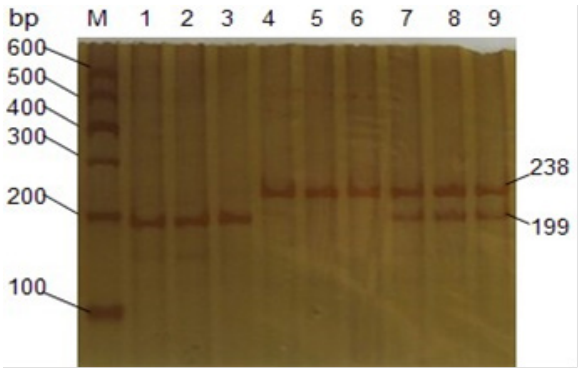

Fig. 1. *Ava*I-RFLP analysis on amplified region by primer P5 of *VEGF* in sheep.
Lanes 1 to 3: genotype CC; lanes 4 to 6: genotype TT; lanes 7 to 9: genotype CT; M: DNA Marker I (TIANGEN, China).

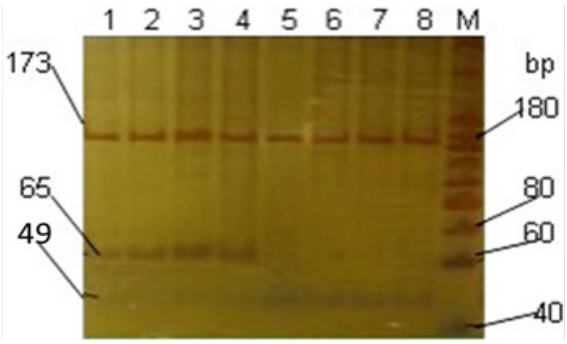

Fig. 2. *Pst*I-RFLP analysis on amplified region by primer P5 of *VEGF* in sheep.
Lanes 1 to 4: genotype GT; lanes 5 to 8: genotype GG; M: 20 bp DNA Ladder Marker (TAKARA).

One enzyme digestion site *Bsa*HI 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).

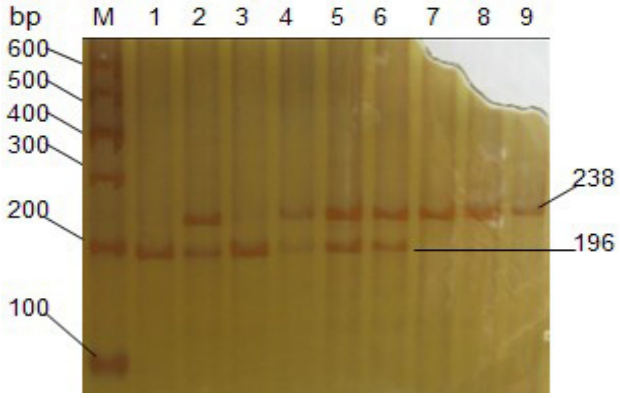

Fig. 3. *Bsa*HI-RFLP analysis on amplified region by primer P5 of *VEGF* in sheep.
Lanes 1 and 3: genotype CC; lanes 2, 4, 5 and 6: genotype CT; lanes 7 to 9: genotype TT; M: DNA.

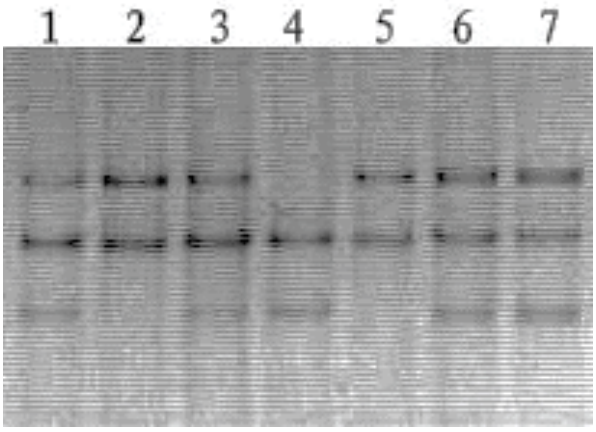

Fig. 4. SSCP analysis of PCR amplification products of primer P1 in sheep *IGF2*.
Lanes 2 and 5: genotype GG; lanes 1,3,6 and 7: genotype GA; lane 4: genotype AA.

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 changes at 14908 bp in the sixth exon (Figs. 5-7), while no polymorphisms was detected in products amplified by primers P1 to P4.

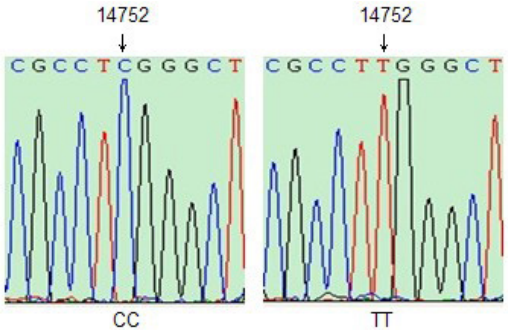

Fig. 5. Partial sequence comparison of CC and TT genotypes located in the 14752 locus of *VEGF* in sheep.

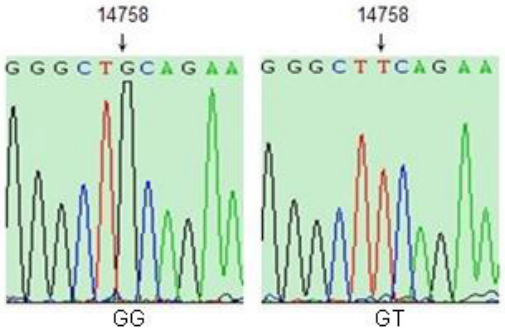

Fig. 6. Partial sequence comparison of GG and GT genotypes at the 14758 locus of sheep *VEGF*.

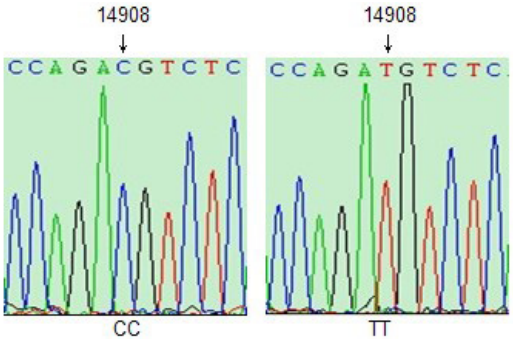

Fig. 7. Partial sequence comparison of CC and TT genotypes at the 14908 locus of sheep *VEGF*.

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.

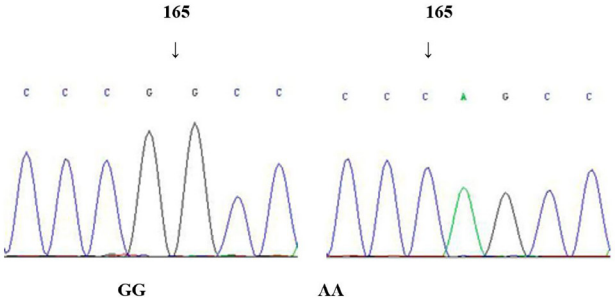

Fig. 8. Sequence comparison of 165 bp of GG and AA genotypes of *IGF2* primer P1 in sheep.

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.

Locus	Genotype	Number	Lambing number
g. 14752 C>T	CC	128	1.81±0.11 [c]
	CT	99	2.16±0.14 [b]
	TT	17	2.86±0.20 [a]
g. *14758* C>T	GG	211	2.00±0.10 [a]
	GT	30	2.25±0.18 [a]
g. *14908* C>T	CC	24	2.16±0.19 [a]
	CT	112	2.08±0.11 [a]
	TT	110	1.95±0.12 [a]

a, b, c Row means with different superscripts differ significantly at $P<0.05$.

Lambing number of small tail Han sheep was significantly influenced by sire, lambing season and parity ($P<0.01$ and $P<0.05$, respectively). The least squares mean and standard error for lambing number of different *IGF2* genotypes in Small-Tail Han sheep are shown in Table IV.

For SNP *IGF2* g. 165 G>A, the small tail Han sheep with genotype AA and GA had 0.47 ($P<0.05$) and 1.12 ($P<0.05$) lambs more than that with genotype GG, respectively. AA had 0.68 lambs more than that with GA ($P<0.05$). No significant difference in lambing number among GG, GA and AA genotypes in small tail Han sheep was observed ($P>0.05$).

Table IV. Least squares mean and standard error for lambing number of different genotypes at three loci of *IGF2* in small tail Han sheep.

Locus	Genotype	Number	Lambing number
g. 165G>A	GG	118	1.45±0.12[c]
	GA	112	1.89±0.14[b]
	AA	43	2.57±0.17[a]

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 pathway by the expression of *VEGF* in luteal cells 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 *IGF*1 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 *IGF*BPs regulate reproductive function as *IGF*s 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 ch*roni*c 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; Carr *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.

ACKNOWLEDGEMENTS

This research was funded by National Natural Science Foundation of China (31772580), China Agriculture Research System of MOF and MARA (CARS-38), Agricultural Science and Technology Innovation Program of China (CAAS-ZDRW202106 and ASTIP-IAS13).

Funding

This research was funded by National Natural Science Foundation of China (32172704), China Agriculture Research System of MOF and MARA (CARS-38), Agricultural Science and Technology Innovation Program of China (CAAS-ZDRW202106 and ASTIP-IAS13).

IRB approval

This study and all the experimental procedures were approved by the Science Research Department (incharge 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.

REFERENCES

Babitha, V., Yadav, V.P., Chouhan, V.S., Hyder, I., Dangi, S.S., Gupta, M., Khan, F.A., Taru Sharma, G., and Sarkar, M., 2014. Luteinizing hormone, insulin like growth factor-1, and epidermal growth factor stimulate vascular endothelial growth factor production in cultured bubaline granulosa cells. *Gen. Comp. Endocrinol*., **198**: 1-12. https://doi.org/10.1016/j.ygcen.2013.12.004

Badinga, L., Song, S., Simmen, R.C., Clarke, J.B., Clemmons, D.R., and Simmen, F.A., 1999. Complex mediation of uterine endometrial epithelial cell growth by insulin-like growth factor-II (IGF-II) and IGF-binding protein-2. *J. mol. Endocrinol*., **23**: 277-285. https://doi.org/10.1677/jme.0.0230277

Bagnicka, E., Siadkowska, E., Strzalkowska, N., Zelazowska, B., Flisikowski, K., Krzyzewski, J., and Zwierzchowski, L., 2010. Association of polymorphisms in exons 2 and 10 of the insulin-like growth factor 2 (IGF2) gene with milk production traits in Polish Holstein-Friesian cattle. *J. Dairy Res*., **77**: 37-42. https://doi.org/10.1017/S0022029909990197

Berkowicz, E.W., Magee, D.A., Sikora, K.M., Berry, D.P., Howard, D.J., Mullen, M.P., Evans, R.D., Spillane, C., and Machugh, D.E., 2011. Single nucleotide polymorphisms at the imprinted bovine insulin-like growth factor 2 (IGF2) locus are associated with dairy performance in Irish Holstein-Friesian cattle. *J. Dairy Res*., **78**: 1-8. https://doi.org/10.1017/S0022029910000567

Botkjaer, J.A., Pors, S.E., Petersen, T.S., Kristensen, S.G., Jeppesen, J.V., Oxvig, C., and Andersen, C.Y., 2019. Transcription profile of the insulin-like growth factor signaling pathway during human ovarian follicular development. *J. Assist. Reprod. Genet*., **36**: 889-903. https://doi.org/10.1007/s10815-019-01432-x

Brioude, F., 2021. Régulation de IGF2 et implications périnatales. *Annls. Endocrinol*., **82**: 5. https://doi.org/10.1016/j.ando.2021.07.037

Cannarella, R., Condorelli, R.A., La Vignera, S., Bellucci, C., Luca, G., Calafiore, R., and Calogero, A.E., 2020. *IGF2* and *IGF1R* mRNAs are detectable in human spermatozoa. *World J. Mens Hlth*., **38**: 545-551. https://doi.org/10.5534/wjmh.190070

Carr, D.J., Wallace, J.M., Aitken, R.P., Milne, J.S., Martin, J.F., Zachary, I.C., Peebles, D.M., and David, A.L., 2016. Peri- and postnatal effects of prenatal adenoviral *VEGF* gene therapy in growth-restricted sheep. *Biol. Reprod*., **94**: 142. https://doi.org/10.1095/biolreprod.115.133744

Carr, D.J., Wallace, J.M., Aitken, R.P., Milne, J.S., Mehta, V., Martin, J.F., Zachary, I.C., Peebles, D.M., David, A.L., 2014. Uteroplacental adenovirus vascular endothelial growth factor gene therapy increases fetal growth velocity in growth-restricted sheep pregnancies. *Hum. Gene Ther*., **25**: 375-384. https://doi.org/10.1089/hum.2013.214

Chen, H.Y., Shen, H., Jia, B., Zhang, Y.S., Wang, X.H., and Zeng, X.C., 2015. Differential gene expression in ovaries of Qira black sheep and Hetian sheep using RNA-Seq technique. *PLoS One*, **10**: e0120170. https://doi.org/10.1371/journal.pone.0120170

De Bock, K., Georgiadou, M., and Carmeliet, P., 2013. Role of endothelial cell metabolism in vessel sprouting. *Cell Metab*., **18**: 634-647. https://doi.org/10.1016/j.cmet.2013.08.001

Ersoy-Canillioglu, Y., and Erkanli-Senturk, G., 2020. Alterations of IL-1 and VEGF after ischemia-reperfusion injured uterus and ovary in rats. *Medeni med. J.* **35:** 106-115. https://doi.org/10.5222/MMJ.2020.67026

Ghosh, D., Najwa, A.R., Khan, M.A., and Sengupta, J., 2011. IGF2, IGF binding protein 1, and matrix metalloproteinases 2 and 9 in implantation-stage endometrium following immunoneutralization of vascular endothelial growth factor in the rhesus monkey. *Reproduction*, **141**: 501-509. https://doi.org/10.1530/REP-10-0475

He, X., Zhang, Z., Liu, Q., and Chu, M., 2019. Polymorphisms of the melatonin receptor 1A gene that affects the reproductive seasonality and litter size in small tail Han sheep. *Reprod. Domest. Anim*., **54**: 1400-1410. https://doi.org/10.1111/rda.13538

Hiden, U., Glitzner, E., Hartmann, M., and Desoye, G., 2009. Insulin and the IGF system in the human placenta of normal and diabetic pregnancies. *J. Anat*., **215**: 60-68. https://doi.org/10.1111/j.1469-7580.2008.01035.x

Higuchi, K., Kazeto, Y., Ozaki, Y., Izumida, D., Hotta, T., Soyano, K., and Gen, K., 2020. Insulin-like growth factors 1 and 2 regulate gene expression and enzymatic activity of cyp17a1 in ovarian follicles of the yellowtail, *Seriola quinqueradiata*. *Heliyon*, **6**: e04181. https://doi.org/10.1016/j.heliyon.2020.e04181

Hsu, C.F., Huang, H.S., Chen, P.C., Ding, D.C., and Chu, T.Y., 2019. IGF-axis confers transformation

and regeneration of fallopian tube fimbria epithelium upon ovulation. *eBioMedicine*, **41**: 597-609. https://doi.org/10.1016/j.ebiom.2019.01.061

Jungerius, B.J., Van Laere, A.S., Te Pas, M.F., Van Oost, B.A., Andersson, L., and Groenen, M.A., 2004. The IGF2-intron3-G3072A substitution explains a major imprinted QTL effect on backfat thickness in a Meishan x European white pig intercross. *Genet. Res*., **84**: 95-101. https://doi.org/10.1017/S0016672304007098

Kawahara, M., Wu, Q., and Kono, T., 2010. Involvement of insulin-like growth factor 2 in angiogenic factor transcription in Bi-maternal mouse conceptuses. *J. Reprod. Dev*., **56**: 79-85. https://doi.org/10.1262/jrd.09-140A

Keshavarzi, F., Shahrakipoor, M., Teimoori, B., Yaghmaei, M., Narooei-Nejad, M., Rasooli, A., and Salimi, S., 2019. Association of the placental VEGF promoter polymorphisms and VEGF mRNA expression with preeclampsia. *Clin. exp. Hypertens*, **41**: 274-279. https://doi.org/10.1080/10641963.2018.1469644

Kona, S.S.R., Kumar, A., Punyakumari, B., Kumar, R.V.S., and Rao, V.H., 2021. Influence of TCM 199B, alpha-MEM, Waymouth MB 752/1 culture media, VEGF, Estradiol-17beta, GDF-9 and FGF on *in vitro* development of preantral follicles in sheep. *Vet. Anim. Sci*., **13**: 100189. https://doi.org/10.1016/j.vas.2021.100189

Li, C., Liu, Z., Li, W., Zhang, L., Zhou, J., Sun, M., Zhou, J., Yao, W., Zhang, X., Wang, H., Tao, J., Shen, M., and Liu, H., 2020. The FSH-HIF-1alpha-VEGF pathway is critical for ovulation and oocyte health but not necessary for follicular growth in mice. *Endocrinology*, **161**: bqaa038. https://doi.org/10.1210/endocr/bqaa038

Lupicka, M., Zadroga, A., Szczepanska, A., and Korzekwa, A.J., 2019. Effect of ovarian steroids on vascular endothelial growth factor a expression in bovine uterine endothelial cells during adenomyosis. *BMC Vet. Res*., **15**: 473. https://doi.org/10.1186/s12917-019-2222-0

Mac Gabhann, F., and Popel, A.S., 2008. Systems biology of vascular endothelial growth factors. *Microcirculation*, **15**: 715-738. https://doi.org/10.1080/10739680802095964

Malysz-Cymborska, I., and Andronowska, A., 2014. Expression of the vascular endothelial growth factor receptor system in porcine oviducts after induction of ovulation and superovulation. *Domest. Anim. Endocrinol.,* **49**: 86-95. https://doi.org/10.1016/j.domaniend.2014.06.003

Nordin, M., Bergman, D., Halje, M., Engstrom, W., and Ward, A., 2014. Epigenetic regulation of the Igf2/H19 gene cluster. *Cell Prolif*., **47**: 189-199. https://doi.org/10.1111/cpr.12106

Olaya, C.M., Michael, F., Fabian, G., Silva, J.L., and Bernal, J.E., Research Seedbed in Perinatal Medicine, and P.U.J.,Garzon, A.L., 2019. Role of VEGF in the differential growth between the fetal and placental ends of the umbilical cord. *J. Neonatal Perinat. Med*., **12**: 47-56. https://doi.org/10.3233/NPM-1795

Park, C.H., Uh, K.J., Mulligan, B.P., Jeung, E.B., Hyun, S.H., Shin, T., Ka, H., and Lee, C.K., 2011. Analysis of imprinted gene expression in normal fertilized and uniparental preimplantation porcine embryos. *PLoS One*, **6**: e22216. https://doi.org/10.1371/journal.pone.0022216

Potente, M., Gerhardt, H., and Carmeliet, P., 2011. Basic and therapeutic aspects of angiogenesis. *Cell*, **146**: 873-887. https://doi.org/10.1016/j.cell.2011.08.039

Rempel, L.A., Nonneman, D.J., Wise, T.H., Erkens, T., Peelman, L.J., and Rohrer, G.A., 2010. Association analyses of candidate single nucleotide polymorphisms on reproductive traits in swine. *J. Anim. Sci*., **88**: 1-15. https://doi.org/10.2527/jas.2009-1985

Roskoski, R., Jr., 2007. Vascular endothelial growth factor (VEGF) signaling in tumor progression. *Crit. Rev. Oncol. Hematol*., **62**: 179-213. https://doi.org/10.1016/j.critrevonc.2007.01.006

Shibuya, M., 2013. Vascular endothelial growth factor and its receptor system: physiological functions in angiogenesis and pathological roles in various diseases. *J. Biochem*., **153**: 13-19. https://doi.org/10.1093/jb/mvs136

Simonetti, A., Rando, A., Di Gregorio, P., Valluzzi, C., Perna, A., and Gambacorta, E., 2018. Variability of the IGF2 locus in the Suino Nero Lucano pig population and its effects on meat quality. *Anim. Prod. Sci*., **58**: 1976-1982. https://doi.org/10.1071/AN17051

Stinckens, A., Mathur, P., Janssens, S., Bruggeman, V., Onagbesan, O.M., Schroyen, M., Spincemaille, G., Decuypere, E., Georges, M., and Buys, N., 2010. Indirect effect of IGF2 intron3 g.3072G>A mutation on prolificacy in sows. *Anim. Genet*., **41**: 493-498. https://doi.org/10.1111/j.1365-2052.2010.02040.x

Suzukawa, A.A., Zanluca, C., Jorge, N.a.N., De Noronha, L., Koishi, A.C., De Paula, C.B.V., Rebutini, P.Z., Nagashima, S., Hansel-Frose, A.F.F., Parreira, V.S.C., Bordignon, J., Macdonald, M.R., Rice, C.M., Passetti, F., and Duarte Dos Santos,

C.N., 2020. Downregulation of IGF2 expression in third trimester placental tissues from Zika virus infected women in Brazil. *J. Infect*., **81**: 766-775. https://doi.org/10.1016/j.jinf.2020.09.028

Tkachenko, O.Y., Wolf, S., Lawson, M.S., Ting, A.Y., Rodrigues, J.K., Xu, F., Bishop, C.V., Stouffer, R.L., and Xu, J., 2021. Insulin-like growth factor 2 is produced by antral follicles and promotes preantral follicle development in macaquesdagger. *Biol. Reprod*., **104**: 602-610. https://doi.org/10.1093/biolre/ioaa227

Wang, X., Yang, Y., and Li, M., 2020. Vascular morphology and expression of angiogenic factors in sheep ovaries and their relation with the lambing rate. *Reprod. Domest. Anim*., **55**: 1132-1138. https://doi.org/10.1111/rda.13751

Wu, A.H., Guo, L.Y., Lu, S., Chen, X.L., Wang, A.A., Wang, X.Y., and Liang, X.F., 2020. Aberrant methylation of IGF2-AS promoter in early pregnancy loss. *Taiwan J. Obstet. Gynecol*., **59**: 109-114. https://doi.org/10.1016/j.tjog.2019.11.017

Younis, S., Naboulsi, R., Wang, X., Cao, X., Larsson, M., Sargsyan, E., Bergsten, P., Welsh, N., and Andersson, L., 2020. The importance of the ZBED6-IGF2 axis for metabolic regulation in mouse myoblast cells. *Fed. Am. Soc. exp. Biol. J*., **34**: 10250-10266. https://doi.org/10.1096/fj.201901321R

Zhang, Z., Huang, Y., Zhang, J., Liu, Z., Lin, Q., and Wang, Z., 2019. Activation of NF-kappaB signaling pathway during HCG-induced VEGF expression in luteal cells. *Cell Biol. Int*., 43: 344-349. https://doi.org/10.1002/cbin.11090