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Research Article

Effect of *Inhibin-βA* Subunit Gene on Reproductive Performance of Kazakh Sheep in Non-breeding Season

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Abstract

Inhibin-βA (INHBA) could feedback suppress synthesis and secretion of follicle-stimulating hormone (FSH), which correlates with the litter size of sheep. In this study, the *inhibin-βA* subunit was used as a candidate gene, and Kazakh sheep was used as a test object. The polymorphism of the gene was detected by PCR-SSCP method and its association with litter size was analyzed. The results showed that there were three polymorphisms in the exon 5'UTR region (primer 0-2), for which AA, AB and BB were detected in Kazakh sheep. The correlation analysis showed that genotype AA had 0.13 ($P<0.05$) lambs and 0.16($P<0.05$) lambs more than genotype AB and BB in Kazakh sheep. Vectors that interfering the *INHBA* expression including PLLU2G-shINHBA-1 (I-1), PLLU2G-shINHBA-2 (I-2), PLLU2G-shINHBA-3 (I-3) and PLLU2G-INHBA-4 (I-4) were constructed by RNA interference (RNAi) technology in the study. After the successfully separated ovarian granulosa cells were transfected with the vector, and the expression level of the gene was detected by quantitative RT-PCR. The results showed that the four vectors suppressed *INHBA* mRNA levels with a silencing efficiency of 34%, 58%, 39% and 19% respectively, with better interference efficiency of I-2. Then, we determined the contents of *INHBA*, FSH, luteinizing hormone (LH) and estradiol (E2) in serum by directing intro-ovarian injection of the I-2. The results showed that the *INHBA* level dropped and the FSH level raised in serum, while LH and E2 levels did not change, indicating the RNAi vector could successfully silence the *INHBA* expression *in vivo*. This study sets a good theoretical basis of researching the breeding and estrus properties of sheep, and the short hairpin (shRNA) vector is hopefully used in promoting the fecundity of sheep in practice.

Keywords: *Inhibin-βA (INHBA), Kazakh sheep, RNA interference (RNAi), Ovarian granular cells, Reproductive Performance*

Üreme Mevsimi Dışında Kazak Koyunlarının Üremeleri Üzerine *İnhibin-βA* Geninin Etkisi

Öz

İnhibin-βA (INHBA), folikül stimule edici hormon (FSH) sentez ve salınımını baskılayabilir ki bu durum koyunlarda yavru sayısı ile ilişkili olabilir. Bu çalışmada, *inhibin-βA* geni aday gen olarak değerlendirilerek Kazak koyunları çalışma materyali olarak kullanıldı. Genin polimorfizmi PCR-SSCP metodu ile belirlendi ve yavru sayısı ile ilişkisi analiz edildi. Elde edilen sonuçlar, Kazak koyununda ekzon 5'UTR bölgesinde AA, AB ve BB için üç polimorfizmin bulunduğunu gösterdi (primer 0-2). Korelasyon analizi, Kazak koyununda genotip AA'da 0.13 kuzu bulunduğunu ($P<0.05$) ve AA genotipinde AB ve BB genotiplerinden 0.16 daha fazla ($P<0.05$) kuzu bulunduğun gösterdi. Bu çalışmada INHBA ekspresyonuna etkiyen PLLU2G-shINHBA-1 (I-1), PLLU2G-shINHBA-2 (I-2), PLLU2G-shINHBA-3 (I-3) ve PLLU2G-INHBA-4 (I-4)'ü içeren vektörler, RNA interferans (RNAi) teknolojisi ile oluşturuldu. Başarıyla ayrılan ovaryum granuloza hücreleri vektörler ile transfekte edildi ve gen ekspresyon seviyesi kantitatif RT-PCR ile belirlendi. Sonuçlar dört vektörün, sırasıyla %34, %58, %39 ve %19 gen susturma etkinlikleri ile *INHBA* mRNA seviyelerini baskıladığını ve daha iyi interferans verimi I-2 olduğunu göstermiştir. *INHBA* ve serum FSH, luteinize edici hormon (LH) ve östradiol (E2) seviyeleri I-2'nin intraovaryan enjeksiyonu sonucu ölçüldü. Sonuçlar, *INHBA* seviyesinin düştüğünü ve serum FSH seviyesinin arttığını, LH ve E2 seviyelerinin ise değişmediğini ve böylece RNAi vektörünün *in vivo INHBA* ekspresyonunu başarıyla susturabildiğini gösterdi. Bu çalışma koyunların üreme ve östrus özelliklerini araştırma amacıyla iyi bir teorik temel oluşturmuştur. Kısa saç tokası (shRNA) vektörü, koyun veriminin arttırılması amacıyla uygulamada kullanılabilir.

Anahtar sözcükler: *İnhibin-βA (INHBA), Kazak koyunu, RNA interferans (RNAi),Ovaryum granular hücreleri, Üreme performansı*

INTRODUCTION

Inhibins were firstly discovered in the testicular extracts in 1932 [1]. Robertson et al.[2] isolated the inhibin molecular weigh of 56.000 which contained two subunits 44.000 and 14.000 Daltons from bovine follicular fluid, inhibin A. At the same year, Ling et al.[3] isolated a 32.000 weighing protein with inhibin activity from porcine follicular fluid which

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contained two subunits 18.000 and 14.000 Daltons, inhibin B. Activin and inhibin proteins are dimeric polypeptide that belong to the TGF-β superfamily of growth and differentiation factors. Activins are formed by the hetero-dimeric combinations of the two subunits (activin A =βA-βA, activin AB =βA-βB and activin B =βB-βB) [3]. Inhibin inhibits FSH secretion from the anterior pituitary and in turn regulates gonadal function and development [4]. And the mutations of *INHBA* gene effects on litter size in sheep significantly [5], and plasma *inhibin* concentrations was related to higher ovulation rates in pigs during follicular phase [6]. Ovaries were recovered from six adult female *Lezhi black goats* and Tibetan goats at 12-24 h after onset of estrus, and used to collect follicles to study cDNA sequence and mRNA expression of *INHA* and *INHBA* genes, and the result showed that base changes in *INHA* and *INHBA* genes resulting in amino acid substitutions may be important in regulating the differential fecundity of these goat breeds as molecular mechanism [7].

In sheep, the length of the oestrus cycle from 13 to 19 days and averages 17 days. Estrus also known as heat period when the ewe is receptive to the ram and will stand for mating. It lasts approximately 24 to 36 h which is influenced by the breed and age of the ewe, the onset of puberty, the presence of the male, and the season [8]. Interestingly though, *INHBA* has a certain relationship with the litter size of sheep, and also probably affect on the estrous cycle through the negative feedback mechanism by suppressing synthesis and secretion of FSH [9]. Several groups have developed transgenic RNAi mice that can produce a gene knockdown phenotype by stably integrated shRNA expression vector *in vivo* [10].

Inhibin has selective inhibition on the synthesis and secretion of FSH, which is crucial for follicular maturation, ovarian development and ovulation. A large number of studies have carried out polymorphism studies on the three subunit genes INHa, INHβA and INHβB and the association analysis of multiple fetal traits [11]. Kristensen et al.[12] studied the local goat inhibin gene, the results showed that the inhibin gene polymorphism significantly affected the number of litters in goats. It is, therefore, necessary to study the relationship between Kazakh sheep inhibin gene polymorphism and reproductive traits.

Ovary granular cell is the maximum cells in follicle. The growth and proliferation of granular cells is one of the most significant sign of the development of follicle [13]. We cultured the granular cell *in vitro* to imitate the growth and development of follicle and the estrous condition. In this experiment, *INHBA* gene which secreted by sheep ovary granular cells was selected as the target gene. The four recombinant vectors was used to interfere the *INHBA* in transfecting the ovarian granular cells. The efficiency of transfection and the interference was tested, then picked out the best interference efficiency recombinant vector. In this study, the method of shRNA was used to interfere the expression in the granular cells of sheep *in vitro* and *in vivo* to explain the mechanism of *INHBA* in the estrous of non-breeding season and discover a way to effect on the *INHBA* expression which could be used in practice to raise the litter size in the future.

MATERIAL and METHODS

Ethics Statement

This study was approved by the Ethical Committee of Animal Experments, Animal Science and Techonology College, Shihezi University (Number: 2015065). All samples were collected in strict accordance with the committee's guidelines. During the experiment, every effort was made to minimize suffering by the animals.

DNA Samples and Lambing Records

Two hundred and thirty genomic DNA samples were obtained from healthy ewes by intravenous blood collection in Xinjiang, China. In this study, a total of 6 Kazakh sheep in good condition were chosen, including 1 sheep deal with saline as one group and 1 sheep deal with empty vectors and 4 sheep deal with interference recombinant vectors. All the Kazakh sheep in the study aged about four years and weighed 49±3.66 kg that were housed individually under the same feeding conditions including *ad libitum* access to alfalfa and water. Genomic DNA was extracted from blood samples using standard phenol-chloroform extraction protocol [14]. Besides that, all lambing records of them were obtained from the production records in the sheep farms.

Primer Design and PCR Amplification

According to the reported sheep *INHBA* gene sequences (NM_001009458.1), two pairs of primers (PD1, and PD2) were designed to amplify the sheep *INHBA* gene. PD1(F: 5'-GGGGAGGAGGCTGAGGAAGT-3' and R:5'-CACA GTAGTTGGCGTGGTAG-3'), PD2(F: 5'-GAGCAGTCGCACAGA CCTTT-3') were separately used to amplify 484 bp, and 196 bp PCR products for 5'-UTR and exon 1 respectively. The primers were synthesized by Xinjiang Kuntailui Co., Ltd. The PCR was performed in a 25 μL reaction mixture containing 0.4 μM of each primer, 200 μM dNTPs, 1× polymerase buffer (including 1.5 mM $MgCl_2$), 1 units of Taq DNA polymerase (Sangon, China) and approximately 100 ng genomic DNA as template. The cycling protocol was 5 min at 95°C followed by 35 cycles of 94°C for 30 s, X°C annealing for 30 s, 72°C for 30 s, with a final extension at 72°C for 10 min (X°C was 58°C and 56°C for PD1, and PD2 primers, respectively)

Single Stranded Conformation Polymorphism (SSCP) and DNA Sequencing Analysis

All PCR products were subjected to SSCP analysis. Aliquots of 2 μL PCR products were mixed with 8 μL loading dye,

denatured by heating at 98°C for 10 min and immediately placed on wet ice. Denatured samples of P1, P2 and P3 were loaded on 10% PAGE gel in 0.5×TBE buffer and constant voltage 140 V for 14-16 h after a pre-run at 220 V for 50 min. The gel was stained by a silver staining method [15].

The 2 PCR products showed different electrophoresis patterns, which were subcloned to pMD19-T vector (Tiangen, China) and sequenced using a commercial service (Huada, Beijing, China). Nucleotide sequence alignments, translations and comparisons were carried out by using DNAMAN software, respectively.

RNAi Vector Construction of INHBA in Sheep

The sequence of RNAi fragment designed in this experiment, depended on the sequence of *INHBA* gene in GenBank (NM_001009458.1) and the principle of RNAi. The construction of the recombination vectors were chosen PLLU2G lentivirus vector. After getting the four RNAi fragments I-1, I-2, I-3 and I-4; the PLLU2G vectors were digested by the *XhoI* and *HpaI* restriction enzymes (Thermo Fisher Scientific, China). The enzyme system: PLLU2G (500 ng/μL) 55 μL, 10×K buffer 5 μL, *XhoI* and *HpaI* 2.0 μL were added to RNase-free water 50 μL at 37°C for 3 h, and tested by 1% agarose gel electrophoresis and collected the vector after digested. PLLU2G linked with the four RNAi fragments (I-1, I-2, I-3 and I-4) over night at 4°C and set the empty control. The recombined vectors transfected the *Escherichia coli* (Thermo Fisher Scientific, China) competent cell overnight. Next day, the monoclonal colony to corresponding resistance LB liquid medium to propagate was picked and tested by PCR, and determined by primers F: AGGCTTAATGTGCGATAAAAGAC, R: GAGCTTATCGATACCGTCGAC, which were sequenced by Invitrogen.

Cell Culture and Transfection Experiment

The ovary was obtained after killing the sheep and the sample was put into the 75% alcohol for 30 s to remove the bacteria from the surface. Later, the samples were washed by PBS (PH=7.2-7.4) for three times and finally the sample was put into the PBS (Invitrogen, Carlsbad, CA, USA) with penicillin and streptomycin. The follicle which is 2-3 mm was pierced through the injection syringe and the liquid from the follicle was taken out into the DMEM (Invitrogen, Carlsbad, CA, USA) with penicillin and streptomycin, which were rinsed repeatedly. The DMEM liquid was filled with the ovary granular cells. The liquid was transferred into the 15 mL centrifuge tube and centrifuge at 1000 rpm for 10 min. The liquid was added in DMEM to suspend the cells and the mixed liquid was filtrated through the 200 meshsieve filtration. After that 5mL 10% fetal bovine serum was added in DMEM culture and the cells were cultured at 37°C and 5% CO_2 incubator in 9 cm petri plate. The cell adherent firmly after 12 h, which were then changed in fresh nutrient solution and cell state was observed. When the primary cells grown to 80%, the nutrient solution was absorbed and digested at 37°C, the process was observed under the microscope until the cell ecptomas vanished. The cell suspension were transferred to new petri plate and cultured at 37°C and 5% CO_2 incubator until the cells grow to 70%. The cells were cryopreserved for later experiment.

The primarily cells in well growth state were used in the transfection experiment. Two controls were setted, one control was added the DMEM and the other control was added the empty vectors equal to the recombinant vectors amount. The four test group were I-1, I-2, I-3 and I-4 interfere vectors *(Table 1)*, and every test group was repeated three times. After the recombinant vectors were transferred the ovary granular cells for 48 h, the transfection efficiency was the best. The fluorescence expression level was observed in granular cells and the number of the cells were recorded under the fluorescence microscope. If the transfection efficiency was up to 20% to 40%, the later experiment could be started.

Cell Total RNA Extraction

The nutrition solution in the 6 well cell culture cluster was discarded and washed and clean by PBS, added the 1 mL Trizol (Invitrogen, Carlsbad, CA, USA) into each well, placed on the ice for 5 min until the cells completely splitting. Added 0.2 mL chloroform to tube, shakes for 15 s, stayed at room temperature for 2 to 3 min, centrifuged with 12000 rpm at 4°C for 15 min. Absorbed the upper clear liquid to a new centrifugal tube, and added the equal volume of isopropyl alcohol after blending, placed at room temperature for 10-30 min, centrifuged with 12000 rpm at 4°C for 15 min. Tested the RNA through the 1% agarose electrophoresis and measured the concentration. The concentration of RNA was measured using a nucleic acid concentration meter (Thermo Fisher, USA), and the ratio of OD260/OD280 was observed to be between 1.8 and 2.0, the later experiment could be started.

Table 1. *INHβA RNAi fragment*

Name	Interference Fragment Sequence	Action Area/bp
I-1	TGCCC TTGCT TTGGC TGAGA GGATT	2-26bp
I-2	CATCG GGACG GAGGG CAGAA ATGAA	317-342bp
I-3	GCTGC ACTTT GAGAT TTCCC AAGAA	404-429bp
I-4	CCATC CGTCT CTTTC AACAG CAGAA	514-519bp

Reverse reaction divided into two steps, the first step system: 10 μL, 5 ×g DNA Eraser Buffer 2 μL, gDNA Eraser 1 μL, total RNA <1 μg, added water to 10 μL, the PCR reaction system: 42°C 3 min, 4°C 10min; the second step system: added the RNase Free dH20, 5 ×PrimeScript Buffer 2 (for Real Time) 4 μL, respectively, RT Primer Mix and PrimeScript RT Enzyme Mix 11 μL, respectively, the PCR reaction system: 37°C 20 min, 85°C 5 s, 4°C 10 min. After those, the gene primer PCR was used for detection.

Transfection of Sheep Ovarian Granulosa Cells with RNAi Plasmid

When the ovarian granulosa cells reached 80%, the dead cells and the medium were washed with sterile PBS (pH=7.2-7.4), and 1 mL of DMEM medium solution (pH=7.2-7.4) was added. Then DMEM solution (pH=7.2-7.4) was added to two 1.5 mL RNase-free tubes, one of which was added with 4 μL of liposome 2000, and the other was added with 1.5 times the mass of RNAi carrier solution equivalent to liposome. Shake the two mixture and let stand for 10 min. The liposome-containing DMEM solution was then added to the DMEM solution of the RNAi carrier, and the mixture was gently mixed and allowed to stand for 30 min. The liposome and RNAi carrier mixed solution was transferred to a cell culture plate and placed in an incubator *(Fig. 1)*.

Sheep INHBA Real-time Fluorescent Quantitative PCR

Using Primer5.0, according to the sheep *INHBA* (GenBank: NM_001009458.1) and *GAPDH* (GenBank: NM_001190390.1) designed real-time fluorescent quantitative PCR primers. Primer sequences were shown in *Table 2*. The PCR reaction system: cDNA 1 μL (100 ng/μL), each of the primer 0.5 μL (0.5 μmol/L), RNAase free H_2O 10.5 μL, PCRmix 12.5 μL, total 25 μL. The PCR procedure: denaturation 94°C 25 s, anneal 57°C 25 s, extend 72°C 25 s, 35 reaction cycles. The PCR electrophoresis test analyzed by gel imaging.

Recombinant Plasmid Transfer by Ovarian Injections

The sheep blood were collected 3 days before the injection of interference vectors and 7 days after injection and the level of INHBA, E_2, FSH and LH in serum related with estrous were measured to explore the relationship between the INHBA and estrous in non-breeding season.

The experimental sheep were fixed in binding frame and removed the abdominal wool. The location with less vessels of abdominal blood were selected to anesthetic and after 5-15 min, the scalpel was used to open the areas of muscle layer and cortex, explored the ovary and fixed it, the 1 mL medical syringe with 400 μL interference vectors were used to inject ovarian surface for a few points. After the injection, the wound were closed by a suture, and applied penicillin and streptomycin powder to the wound. The eating and exercise of sheep were observed over night and collected 3 mL blood from the sheep in good condition, kept 3 h at 38°C, centrifuged with 3000 rpm for 2 min and collected the upper clean liquid. Enzyme linked immunosorbent assay (ELISA) were used in the determination of serum INHBA, FSH, LH and E_2 levels. The antibody was diluted to a protein content of 1 to 10 μg/mL with 0.05 M PH=9.0 carbonate coating buffer, and 0.1 mL was added to the reaction well of each polystyrene plate. Subsequently, 0.1 mL of a sample to be tested diluted with a certain amount was added to the reaction well, and the mixture was incubated at 37°C for 1 h. After washing, 0.1 mL of freshly diluted enzyme-labeled antibody was added, and the mixture was incubated at 37°C for 0.5 to 1 h and then washed. 0.1 mL of the prepared TMB substrate solution was added, and the mixture was allowed to stand at 37°C for 10 to 30 min. Finally, 0.05 mL of 2 M sulfuric acid was added to each reaction well to terminate the reaction.

Statistical Analysis

Differences in haplotype frequencies were analyzed using a

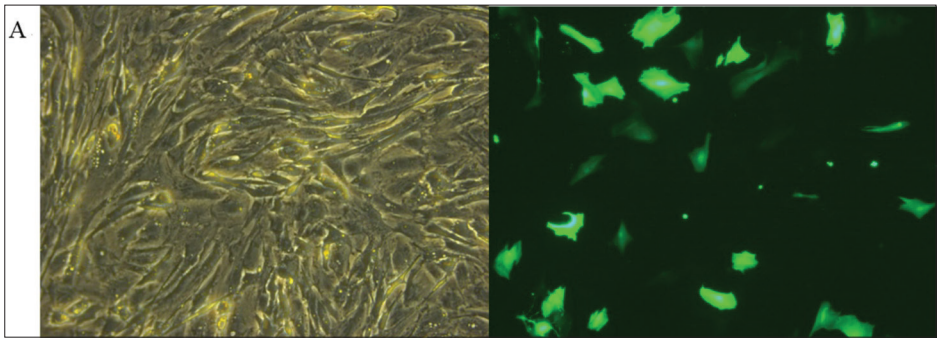


Fig 1. Culture and transfection of ovarian granulosa cells

Table 2. *GAPDH* and *INHBA* RT-PCR primers

Gene Name	Primer Sequence(5'~3')	Amplification Length/bp	The Annealing Temperature/°C
GAPDH	F:TTCTGCTGACGCTCCCA R:CCTCCACGATGCCAAAG	134	57
INHBA	F:CCTCAAGTCGTGCTGTGTG R:GTCTTCGTGTCACCACTGTCT	187	57

χ2-test. The association between polymorphisms in INHBA gene and the number of lambs in serum and the data of the real-time PCR were evaluated using One-Way ANOVA test. The Data were expressed as the mean±the standard error and all statistical analysis were performed with SPSS for Windows (version 20.0).

RESULTS

SSCP analyses of the PCR-amplified fragments in our study from the 484-bp section of the 5'-UTR of the *INHBA* gene showed three distinct banding patterns in Kazakh sheep (n = 230), including AA, AB and BB *(Table 3)*. However there is no primers 1-1 designed for exon 1 were found to have polymorphic sites of amplified fragments. We used the sequences of the full-length INHBA gene of sheep available in GenBank (accession numbers 443524) as a reference to compare with the sequences we obtained from Kazakh sheep. And subsequent sequence comparison revealed there were two SNPs of the PD1 fragments in Kazakh sheep, including g.79A > G and g.107G > A, which the former was changed to AA- and AB-genotypes and the latter was changed to BB- and AB genotypes. Above all, in our study, it is worth to note that Kazakh sheep is a typical single breed. The genotype frequency and allele frequency of INHBA gene in sheep were shown in *Table 4*, AB-genotype frequency of Kazakh sheep was higher than AA and BB, which showed A-genotype was the predominant allele in all the populations.

Polymorphism information content (PIC), population heterozygosity, effective allele number, and χ2-test results of loci in Kazakh sheep group were shown in *Table 5*. PIC of the populations in Kazakh sheep was separately 0.373 that was ranged within 0.25-0.50, which indicated that the loci was moderately polymorphic in the populations.

According to the statistical results in *Table 3*, which can be considered that there is a significant difference in the number of litters between the Kazakh foreign genotype AA and the AB and BB genotypes (P<0.05). In addition, there was no significant difference in the number of lambs between AB genotype and B genotype (P>0.05).

The colony PCR products including two parts: The length of the positive clone was about 302bp, and the negative clone was about 239bp. Taken the positive clone to sequence to define the base did not mutate, and the results of sequencing are showed in the *Fig. 2*. The *Fig. 2A* was the sequence of the origin RNAi fragments and the *Fig. 2B* was the result of sequencing, both of which showed the two sequencing results were exactly same. According to the results, it indicated that there was no mutated base in the recombinant RNAi vector which could be used in the next ovary granular cell transfection experiment.

The ovarian granular cells were into oval or round just after separation, and the cells were adherent 4-6 h later, and the cells connected with the others by extended filiform pseudopodia, the nucleus was large, the cytoplasm appeared a large number of particles, a single-layer adherent growth and divided fast, and after 96 h of separation the dish was covered. After the petri dish was completely covered, the cells present different shapes such as spindle, irregular triangle, polygon or fan. After 48 h of the transfection of ovarian granular cells with recombinant RNAi vectors, it showed that the transfection effect got the peak and the cells in the 6-well cell culture plate covered about 80% and the transfected fluorescent efficiency was about 40% in the transfected groups and finally reached the basic demands of the cell transfection experiment by observing the fluorescent expression under the fluorescent inverted microscope.

Table 3. *Correlation analysis of INHBA with litter size in sheep*

Group	No.	Genotype	The Number of Lamb
Kazakh sheep	230	AA(82)	1.25±0.06[a]
		AB(114)	1.12±0.03[b]
		BB(34)	1.09±0.08[a]

Table 4. *Data of Heterozygosity (H), Effective number of Alleles (Ne), PIC and χ2-test*

Group	H	Ne	PIC	χ^2-test
Kazakh sheep	0.497	1.987	0.373	2.398 (P<0.05)

Table 5. *The genotype frequency and gene frequency of INHBA gene in Kazakh sheep*

Group	No.	INHBA Primer				
		Genotype Frequency			Allele Frequency	
		AA	AB	BB	A	B
Kazakh sheep	230	0.357(82)	0.496(114)	0.147(34)	0.553	0.447

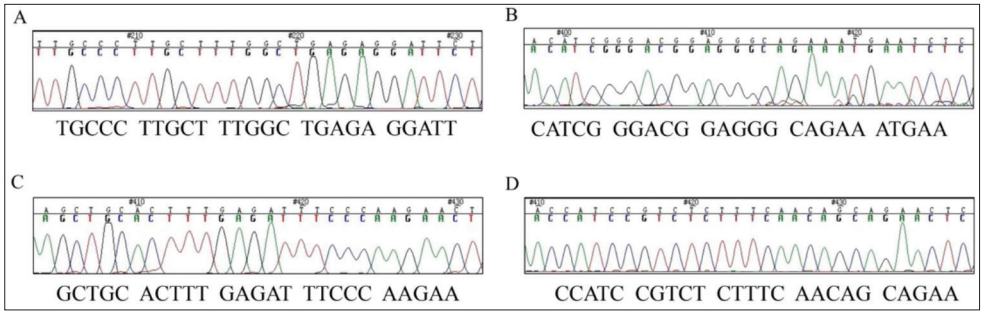
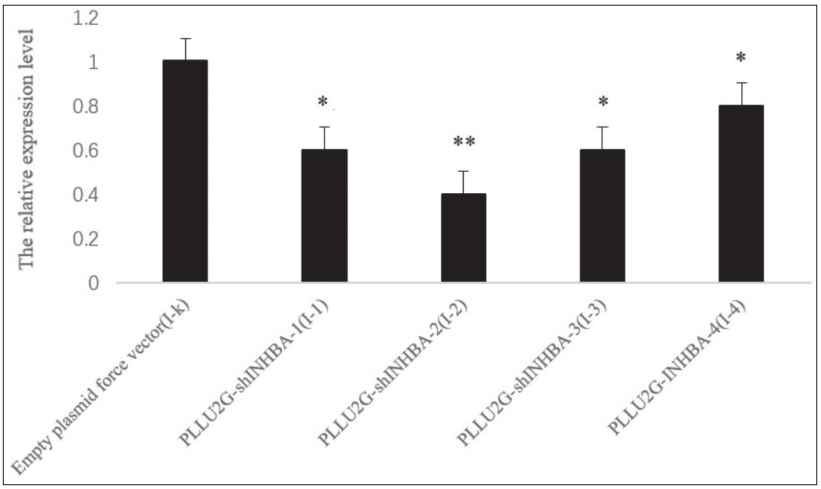


Fig 2. Sequences comparison of recombinant vectors with mRNA of *INHBA*

Fig 3. *INHBA* gene mRNA expression in the ovarian granular cells of sheep



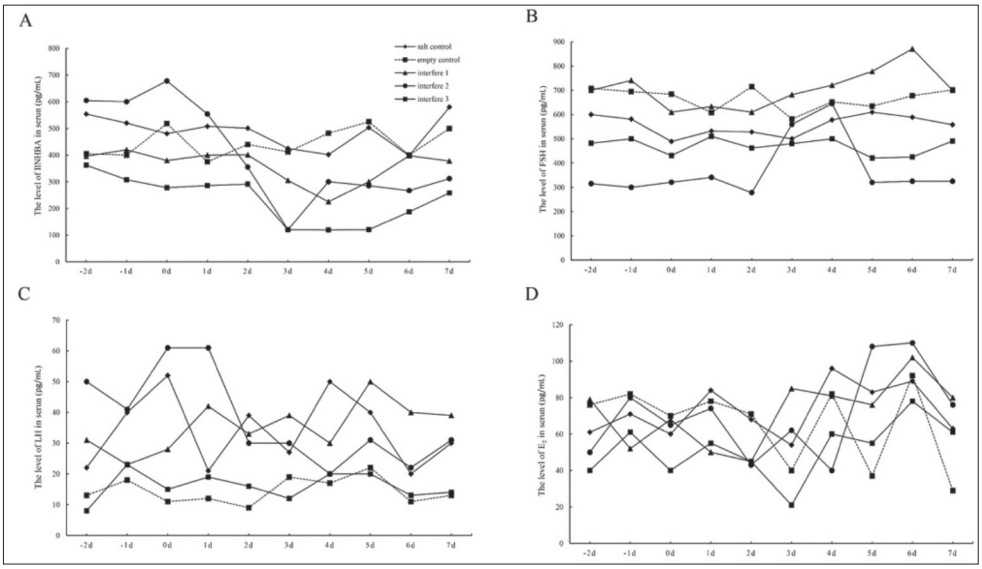


Fig 4. The level of INHBA, FSH, LH and E_2 in serum of sheep

Above all, compared to the empty plasmid force vector (I-K) group, the restructuring plasmid carrier interference on the expression of *INHBA* gene in the ovarian granular cells showed the interference effect, and those relative expression of *INHBA* gene in I-1, I-2, I-3 and I-4 groups after transfection were 0.66, 0.42, 0.62 and 0.66 respectively *(Fig. 3)*, and in turn their interference efficiency were 34%, 58%, 39% and 58%, respectively.

From the *Fig. 4A*, the INHBA level in the salt control and empty control were not changed significantly which keep at the level of 400 pg/mL to 500 pg/mL. However, in the interference group 1, after interference vectors injection, the INHBA level changed at the 2nd day and decreased to the lowest point of 200 pg/mL at the 4th day, then the level began to raise smoothly until the 7th day and the level recovered to 390 pg/mL just as the level before interference. In the interference group 2, the INHBA level dropped sharply at the 1st day after injection until the 3rd day up to the lowest point of 100 pg/mL, then raised gradually. In the interference group 3, the INHBA level decreased to the bottom at the 2nd day and kept at the

bottom of 110 pg/mL for two days and followed shot up. From the *Fig. 4B*, the FSH level in the salt control and empty control was not changed significantly. But in the interference group 1, the level of FSH took off at the 3rd day after injection and reached the peak about 880 pg/mL at the six day, then dropped to the level of 700 pg/mL just as the level before the injection. In the interference 2, the level of FSH raised sharply at the 3rd day of 600 pg/mL and it descended again to the level before injection of about 300 pg/mL. In the interference group 3, the level of FSH did not change significantly. Besides those, more interestingly, in the *Fig. 4C* and *Fig. 4D*, we also noted that there were no significant difference between the level of LH and E_2 before and after the interference recombinant vectors injection, which indicated that the INHBA level was lower in the ovaries and it couldn't lead to the changes of LH and E_2 levels in the ovary following the change of INHBA level. Therefore, those results in the study suggested that the interference recombinant vectors could successfully interfere on the INHBA level in the ovarian granular cells of sheep, and according to those results appeared that the FSH raised following the decreasing level of INHBA and the level of LH fall down as the INHBA raised but the level of E_2 raised along with the INHBA raised.

DISCUSSION

Inhibin is a glycoprotein hormone secreted by the gonads, which can inhibit the synthesis and secretion of FSH in the pituitary [13]. Genetic characteristics of single nucleotide mutation in this study were analyzed in the 5`-UTR and exon 1 of INHBA gene in Kazakh sheep. There were three mutation sites of PD1 site in INHBA, by comparing with the reported ovis INHBA sequences, respectively, among which AA genotype with an A → G mutation in 79bp and BB-genotype with a G → A mutation in 107bp were cause amino acid changes. In this study, we selected *INHBA* gene in Kazakh sheep breeds to study the genotype frequency and gene frequency of INHBA gene, which indicated that A-allele could be the predominant gene in the Kazakh sheep populations and it might provide a choice for selecting the sheep breeds. In the previous studies, there were only few reports about the impact of *INHBA* Gene on animal reproductive traits. In the study of the genetic variation of inhibin A gene and the double lamb trait, Chu et al.[14] found that statin is associated with the double lamb trait. In this study, the inhibin βA gene was studied as a candidate gene associated with lamb litter size in sheep. The result showed that the number of Lambs in AA genotypes of Kazakh sheep was significantly higher than that of AB and BB genotypes ($P<0.05$). There was no significant difference in BB and AB genotypes ($P>0.05$). The number of Lambs in AA genotypes in the 5'-UTR of Kazakh sheep was significantly higher than that in genotypes AB and BB, indicating that allele A is a dominant allele relative to allele B, to a certain extent. It indicates that the homozygous AA mutation in this experiment is positively correlated with the number of lambs in Kazakh sheep in Xinjiang, which is worthy of further study.

Inhibins are produced and secreted by the granulosa cells of the largest follicles during terminal follicular development and is important inhibitors of FSH secretion by pituitary gonadotrophs. A study showed that *INHBA* knock-out mouse exhibited reduced expression of *TGFBR3*, which disrupted *Inhibin-TGFBR3* signaling and therefore increases the secretion of FSH in the mouse anterior pituitary cells [15]. More interestingly, some studies also showed that the lower INHBA levels could lead to the development of a greater number of follicles in lamb, and attenuate INHBA activity could result in higher fertility and ovulation rates in sheep, cattle, and rats using the INHBA vaccines [12].

RNAi possesses post-transcriptional gene silencing mechanism and high specificity, different from traditional DNA level gene knockout technology. In the present study, in order to explore the correlation of INHBA with the reproductive traits in sheep, the RNAi technology was selected and used to construct the recombinant vectors with effective shRNA fragment, and the latter was performed well in the transfection of granular cells *in vitro* and in the ovary of sheep. Han et al.[16] reported that RNAi could inhibit virus production and effectively inhibit pre- and/or post-integration infection events in the HIV-1 life cycle. Si et al.[4] study showed that the H460 cells at 70% confluence was transfected using the optimal small interfering (siRNA) and the *INHBA* knockdown efficiency was 93% by the quantitative RT-PCR and the concentration of *INHBA*-targeting siRNA reduced 6% after 96 h of transfection. However, in the study, we constructed four inducible *INHBA* expression vectors as the experiment groups contain I-1, I-2, I-3, I-4, and an empty vector PLLU2G as the negative control group. The results of the study showed that all the silencing efficiency in the four vectors with inhibiting *INHBA* mRNA expression were 34%, 58%, 39% and 19% respectively, which showed that the better interference efficiency was the I-2 group. Therefore, our results also indicated that the I-2 inducible siRNA expression system could efficiently induce conditional inhibition of *INHBA*, which could conditionally inhibit the expression of *INHBA* in not only established stable clones but also transient transfection cells and finally greatly increase its usefulness and convenience [17]. Beside that, in the study, all the interference recombinant vectors were injected into the follicle of sheep, and then measured the serum INHBA levels in different groups, all of which proved that the INHBA indeed had a closed relationship with the steroid hormones, including FSH, LH and E_2, and further effect on the estrous cycle and reproductive performance in sheep, especially in the non-breeding season.

In conclusion, The PLLU2G vector is based on a well-established and can be used shRNA system, combining the puromycin and eGFP markers on a MSCV-based backbone.

The RNAi vector were successfully constructed and transfected into the follicular granular cells of sheep and then silenced the *INHBA* expression *in vivo*, those results presented in this study clearly indicate that this inducible siRNA expression system could efficiently, conditionally and reversibly inhibit *INHBA* expression in sheep. In a word, this study could be used to set a good theoretical basis of researching the breeding properties and estrus properties of sheep, and the shRNA vector is hopefully used in promoting the fecundity of sheep in practice.

Competing Interests

There are no potential conflicts of interest.

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