

Cloning and Tissue Expression of the *TTR* Gene During the Initiation of Puberty in Dolang Sheep

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ABSTRACT

To study the expression level and function of the *TTR* gene in different tissues of Dolang sheep at the periods of prepuberty, puberty and postpuberty, we collected hypothalamic, pituitary, ovarian, oviduct, and uterine tissues. The *TTR* gene was amplified, cloned, and sequenced using RT-PCR in Dolang sheep. Its expression in the five tissue types was analyzed. The amino acid sequences of the *TTR* gene were compared to those of other species and a phylogenetic tree was produced. qPCR was used to detect the expression levels of the *TTR* gene in the tissues. *TTR* gene was transfected into granulosa cells of the ovaries of Dolang sheep and was detected by fluorescence quantification. The cDNA sequence of the *TTR* gene of Dolang sheep was 444 bp and encoded 147 amino acids. Amino acid sequence identity of *TTR* from Dolang sheep was 99.32%, 99.32%, and 93.20% with sheep, goat, and cattle, respectively, and 89.33%, 89.12%, and 86.39% with pigs, horses, and humans, respectively. The *TTR* gene was expressed in all five tissue types, with higher expression in the pituitary and the hypothalamus ($P < 0.05$). *TTR* gene expression in the hypothalamic tissues was significantly higher at the periods of puberty than prepuberty and postpuberty ($P < 0.05$). The expression of *TTR* genes in the pituitary and uterus increased significantly from puberty to postpuberty ($P < 0.05$). Changes of *TTR* gene expression in the hypothalamus, pituitary, and uterus at different pubertal stages suggest that *TTR* genes may be involved in the initiation of puberty in sheep. The *TTR* gene has a repressive effect on the expression of the *GnRH* gene and may indirectly regulate the initiation of puberty in Dolang sheep. Our results provide a new theoretical basis for studying the initiation of puberty in sheep.

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Authors' Contribution

ZZ conceived the study, conducted data analysis, and prepared the figures and tables. ZZ, ZS, QL, and JZ performed the sample collection and total RNA preparation. ZZ, YZ, CW, and XL performed the qRT-PCR validation. ZZ prepared the manuscript. FX is the corresponding author and provided financial support for this study. All authors have read and approved the final manuscript.

Key words

Puberty, GnRH, cDNA, Expression, TTR

INTRODUCTION

Puberty is the period of first estrus and ovulation in females and is associated with the regulation of the hypothalamic-pituitary-gonadal axis and the interaction of environmental and genetic factors on the coordinated function of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Meeran *et al.*, 2003; Pool *et al.*, 2020; Redmond *et al.*, 2011; Rosa and Bryant, 2003;

Wankowska *et al.*, 2008). The gonadotropin hypothesis suggests that as the body develops, hypothalamic gonadotropin-releasing hormone (GnRH) neurons become less sensitive to negative feedback effects, thus allowing for increased GnRH secretion, which stimulates gonadotropin secretion and ultimately leads to follicle development and ovulation (Day and Anderson, 1998). Normal or disturbed pubertal development is mainly determined by genetic factors (Gajdos *et al.*, 2010; L and R, 2005).

Transthyretin (TTR) is a 56 kDa protein consisting of four homogeneous subunits of 14 kDa each and is one of the three steroid hormone distribution proteins (THDP) in mammals (Schreiber, 2002). The locus encoding human thyroid TTR is mapped to chromosome 18 (Wallace *et al.*, 1985), while the gene encoding porcine TTR is localized to chromosome 6 (Archibald *et al.*, 1996). It binds and transports thyroid hormones in the blood, together with thyroxine-binding globulin (TBG) and albumin (Zhan *et al.*, 2006). It has been shown that estrogens regulates

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TTR expression in mouse liver and choroid plexus through estrogen receptors (Goncalves *et al.*, 2008). Other hormones such as progesterone 5 α -dihydrotestosterone and glucocorticoids, also regulate *TTR* expression (Martinho *et al.*, 2012; Quintela *et al.*, 2008, 2011). It was upregulated by sex hormones in mice liver (Goncalves *et al.*, 2008). In a previous study, it was reported that *TTR* gene expression in the hypothalamic tissue of Dolang sheep was significantly lower in prepuberty and postpuberty than in puberty (Zhang *et al.*, 2022). It has been suggested that the negative feedback effect of progesterone on GnRH release may be mediated by *TTR*, which decreases the probability of IGF1 binding to its receptor, thus inhibiting GnRH activity (Zhang *et al.*, 2020). Since GnRH has a facilitative effect on the initiation of puberty in mammals (Garcia *et al.*, 2018), we hypothesized that *TTR* may indirectly regulate the initiation of puberty in Dolang sheep through the regulation of GnRH.

Dolang sheep is a typical representative of early pubertal breeds in Xinjiang, reaching puberty in only 3–4 months (Xing *et al.*, 2019). We investigated the sequence of the *TTR* gene, its tissue expression, and its regulatory role in the *GnRH* gene to investigate the effect of the *TTR* gene on pubertal initiation in Dolang sheep.

MATERIALS AND METHODS

Animal pubertal identification and sample collection

Tissues from prepubertal, pubertal, and postpubertal Dolang sheep were collected. Three biological replicates were performed for each period and analyzed. A total of nine Dolang sheep were in healthy and under the same feeding conditions. Puberty was evaluated using the following criteria: excitement, sensitivity to external stimuli, frequent urination, red and swollen vulva with mucus, and

standing still to accept ram climbing. Fresh tissue samples were collected from five parts of hypothalamus, pituitary, oviduct, ovary, and uterus after slaughter, and all samples were stored at -80°C. The sheep used in the experiment were obtained from the experimental station at Tarim University, China.

Primer design and synthesis

The primers were designed using Primer Premier 5.0 software with reference to the sequences of *TTR* (accession number: NM_001009800.1), *IGF1R* (accession number: XM_027957015.2), and *GnRH* (accession number: XM_015093089.3) of sheep in GenBank; primers for gene cloning and real-time fluorescent quantitative PCR are shown in Table I.

Total RNA extraction, reverse transcription and gene cloning

Total RNA was extracted from each tissue according to the TRIzol method, and the integrity of the RNA bands was examined using 1.0% agarose gel electrophoresis. The cDNA was synthesized by reverse transcription using PrimeScript™ RT reagent kit and stored at -20°C. The *TTR* gene was amplified using the *TTR* primer (Table I) with cDNA from the hypothalamic tissue as the template. A total of 25 μ L of PCR reaction system premix 12.5 μ L, 1 μ L each of 10 μ mol/L forward and reverse primers, 1 μ L cDNA, 9.5 μ L ddH₂O was further added. The PCR amplification procedure used was 5 min preheating at 95°C, 30 s denaturation at 95°C, 30 s annealing at 58°C, 30 s extension at 72°C, total 38 cycles, 5 min extension at 72°C, storage at 4°C. The PCR product size was determined using 1.0% agarose gel electrophoresis.

Table I. Primer information.

Genes	Primer sequences (5'→3')	Annealing temperature/°C	Product size/bp	Applications
<i>TTR</i>	F: TTCTTGGCAGGATGGCTTC R: TTTT TTTT TTTT TTTGCTTTAATAGGCATGTTTATTG	58.0	610	RT-PCR
<i>Q_TTR</i>	F: TGTCTCTGATGGTCAAGGTCCTG R: TCGTCAGCAGCCTTCTTGAACAC	56.5	89	Q-PCR
<i>β-actin</i>	F: TTCCAGCCTTCCTTCCTG R: CCGTGTGTCGTAGAGGT	56	109	Q-PCR
<i>Q_IGF1R</i>	F: AACATCGCTTCGGAAGTGGAGAAC R: TCAGGAAGGACAAGGAGACCAAGG	60	103	Q-PCR
<i>Q_GNRH</i>	F: CCCTGGAGGAAAGAGAAATGCTGAG R: GCACTTAGGTTCTACTGGCTGATCG	59.7	88	Q-PCR

Table II. Bioinformatics software.

Software	Websites	Applications
Mega 5.0		Constructing phylogenetic trees
ProtParam	https://web.expasy.org/cgi-bin/protparam/protparam	Physicochemical properties of proteins
ProtScale	https://web.expasy.org/cgi-bin/protscale/protscale.pl?1	Hydrophobicity of proteins
TMHMM	http://www.cbs.dtu.dk/cgi-bin/webface2.fcgi?-jobid=611A2DE000005671E42EBF66andwait=20	Protein transmembrane structural domains
SignalP 4.0	http://www.cbs.dtu.dk/cgi-bin/webface2.fcgi?-jobid=611A3037000058DD235450D5andwait=20	Protein signal peptide prediction
NetPhos 3.1	http://www.cbs.dtu.dk/cgi-bin/webface2.fcgi?jobid=611B-17CB000009EDCC024BDCandwait=20	Protein phosphorylation sites
SOPMA	https://npsa-prabi.ibcp.fr/cgi-bin/secpred_sopma.pl	Protein secondary structure prediction

The PCR products were recovered using an agarose gel DNA recovery kit (TIANGel Midi Purification Kit), ligated with pMD19-T vector, transformed the ligated products into *E. coli* DH5 α receptor cells, shaken for 50 min at 37°C in a shaker, and then incubated on LB solid medium containing ampicillin (Amp). After colony growth, positive colonies were selected and identified by PCR, and the correctly identified bacterial broth was sent to Biotech Bioengineering (Shanghai) Co for sequencing.

Bioinformatics analysis of the sequence coding for amino acids in protein of TTR gene

The amino acid sequences encoded by the *TTR* gene of Dolang sheep were compared with those of domestic sheep (*Ovis aries*, accession number: NP_001009800.1), goat (*Capra hircus*, accession number: XP_005697069.1), cattle (*Bos taurus*, accession number: NP_776392.1), pig (*Sus scrofa*, accession number: NP_999377.1), horse (*Equus caballus*, accession number: XP_001495232.1), and humans (*Homo sapiens*, accession number: NP_000362.1) for similarity, and the corresponding sequences of the *TTR* gene of Dolang sheep were analyzed bioinformatically using online software (Table II).

TTR gene tissue expression analysis

Real-time fluorescent quantitative PCR (qPCR) analysis was performed using cDNA from five tissues of the hypothalamus, pituitary, fallopian tube, ovary, and uterus of Dolang sheep as templates. A total of 15 μ L of PCR reaction system was added, which included ddH₂O 5.5 μ L, PerfectStart Green qPCR SuperMix (2 $\times$) 7.5 μ L, cDNA 1 μ L, 0.5 μ L each of upstream and downstream primers. PCR reaction procedure followed was pre-denaturation at 95°C for 15 s; denaturation at 95°C for 15 s, annealing at 56.5°C for 15 s, extension at 68°C for 20 s, consisting of 40 cycles in total.

Construction of expressing vector of fusion gene of TTR-EGFP

The recombination expression vector was constructed as follows. The pEGFP-N1 vector was digested with NheI and EcoRI. The 444 bp CDS region of the *TTR* gene was amplified from the *TTR* cDNA of Dolang sheep using RT-PCR. The amplified cDNA was digested with NheI and EcoRI, ligated into the pEGFP-N1 vector to generate pEGFP-N1-TTR, and verified by sequencing.

Cell culture and fusion gene transfection

Ovarian granulosa cells were cultured in DMEM medium (thermofisher biochemical products (Beijing) co., Ltd.) containing 10% fetal bovine serum (Zhejiang Tianhang Biotechnology Co., Ltd.), 1% double antibiotics (penicillin-streptomycin) (Solarbio) under humidified air containing 5% CO₂ at 37°C. When the cells reached 90% growth, transfection was performed using the Lipofectamine 3000 Transfection Kit, according to the manufacturer's instructions. In the control group, pEGFP-N1 was transfected into cells, and in the test group, pEGFP-N1-TTR was transfected into cells. After 24 h, cellular RNA was extracted using the TRIzol method, and the *TTR* gene transfection into the cells was detected using fluorescence detection and qRT-PCR methods. Detection of *TTR* gene expression in cells 24h, 48h and 72h after transfection.

Effect of TTR gene overexpression on IGF1R and GnRH gene expression.

Because the *GnRH* gene is associated with the initiation of puberty in mammals (Wojniusz *et al.*, 2011), the *TTR* gene can reduce the probability of IGF1 binding to its receptor, thus inhibiting GnRH activity (Hiney *et al.*, 2009; Vieira *et al.*, 2015). Therefore, we examined changes in the expression of *IGF1R* and *GnRH* genes

in ovarian granulosa cells after the introduction of the *TTR* gene. The expression of *IGF1R* and *GnRH* genes in ovarian granulosa cells 24 h after transfection with the *TTR* gene was detected using qRT-PCR. Gene expression was calculated by the $2^{-\Delta\Delta CT}$ method. The data were statistically analyzed using SPSS software (version 22.0), and the significance of differences between two groups was compared by independent samples t-test. Comparisons of three groups and above were tested by one-way ANOVA. Statistical significance was set at $P < 0.05$.

RESULTS

Cloning and sequencing of the TTR gene in Dolang sheep

The target band size of the *TTR* primer was approximately 610 on 1.0% agarose gel electrophoresis, which was consistent with the expected target fragment size (Fig. 1). Sequencing of the product obtained from the cloning of the *TTR* gene of Dolang sheep yielded a sequence size of 610 bp, including 5'UTR 11 bp, 3'UTR 155 bp, and CDS region 444 bp, which was consistent with the expected results.

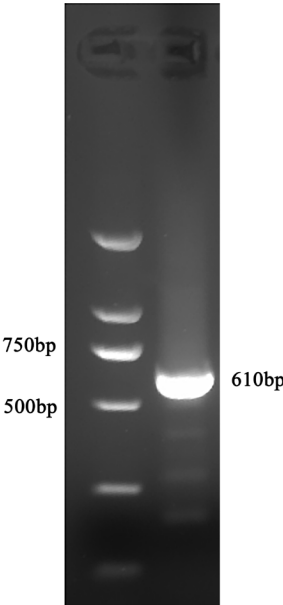


Fig. 1. PCR product of *TTR* gene.

Comparison of amino acid sequence identity of TTR gene and construction of phylogenetic tree

The amino acid sequences of the *TTR* gene of Dolang sheep were 99.32%, 99.32%, 93.20%, 89.33%, 89.12%, and 86.39% similar to those of sheep, goats, cattle, pigs, horses, and humans, respectively (Fig. 2). Based on the amino acid sequences of the CDS region of the *TTR* genes

of different species, the neighbor joining (NJ) tree was constructed using Mega 5.0. As shown in Figure 3, Dolang sheep were more closely related to sheep, goats, and cattle, and more distantly related to pigs, horses, and humans.



Fig. 2. Amino acid similarity alignment of *TTR* protein in Dolang sheep

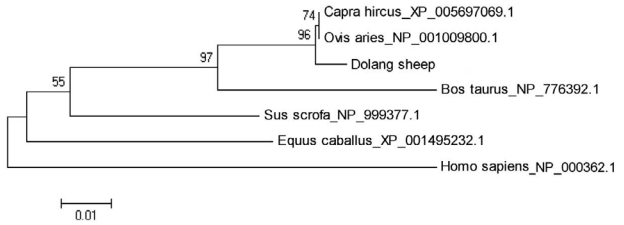


Fig. 3. Phylogenetic tree of *TTR* gene.

Structural and functional prediction of the protein encoded by the TTR gene

Analysis of the physicochemical properties of the protein encoded by the *TTR* gene of Dolang sheep using the online analysis software ProtParam showed that the molecular mass of the protein was 15801.01 u, the theoretical isoelectric point pI was 5.59, the molecular formula of the protein was $C_{716}H_{1113}N_{177}O_{217}S_4$; the total number of atoms was 2227, and a total of 147 amino acids were encoded (Table III), among which leucine (L) and serine (S) both had 17, the highest proportion, both accounting for 11.2%, and glutamine (Q) was not found. The total number of negatively charged amino acid residues (Asp and Glu) was 17. The total number of positively charged amino acid (Arg+Lys) residues was 14, and the

instability coefficient was 38.45. The hydrophobicity of the TTR protein was predicted using the online analysis software ProtScale, which showed that Figure 4a, the regions with scores greater than 0 were denser than those with scores less than 0. The aliphatic index was 86.26. The half-life was 30 h (mammalian reticulocytes *in vitro*), and the grand average of hydropathicity (GRAVY) was 0.036, indicating that the protein is a stable amphiphilic acidic protein. The prediction of the transmembrane structural domain of the Dolang sheep TTR protein using TMHMM software showed that the TTR protein does not contain a transmembrane structural domain (Fig. 4b). Prediction of the signal peptide of the TTR protein from Dolang sheep using the online software SignalP 4.0 revealed that the TTR protein contains a signal peptide with a shear site located between amino acids 19 and 23 (Fig. 4c). The phosphorylation sites of TTR proteins from Dolang sheep were predicted using the online software NetPhos 3.1, which showed a total of 18 phosphorylation sites, including 13 serine (S) sites, four threonine (T) sites, and one tyrosine (Y) site (Fig. 4d). The prediction of the secondary structure of the TTR protein using SOPMA software showed that the protein contained 157 α -helices (24.49%), 42 extended chains (28.57%), eight β -folds (5.44%), and 61 irregular coils (41.50%); α -helices, extended chains, and irregular coils were found throughout the amino acid chain with only a few β -folds (Fig. 5).

Table III. Amino acid composition of TTR protein in Dolang sheep.

Amino acids	Quantity	Proportion/%	Amino acids	Quantity	Proportion/%
Ala (A)	15	10.2	Leu (L)	17	11.6
Arg (R)	3	2.0	Lys (K)	11	7.5
Asn (N)	2	1.4	Met (M)	2	1.4
Asp (D)	6	4.1	Phe (F)	7	4.8
Cys (C)	2	1.4	Pro (P)	7	4.8
Gln (Q)	0	0.0	Ser (S)	17	11.6
Glu (E)	11	7.5	Thr (T)	10	6.8
Gly (G)	11	7.5	Trp (W)	2	1.4
His (H)	3	2.0	Tyr (Y)	6	4.1
Ile (I)	2	1.4	Val (V)	13	8.8

Expression analysis of TTR in different tissues of Dolang sheep at different stages of puberty

TTR gene expression was significantly higher in the hypothalamus than in other tissues ($P < 0.05$). TTR gene expression in the hypothalamic tissues was significantly higher at the period of puberty than prepuberty and

postpuberty ($P < 0.05$). The expression of TTR genes in the pituitary and uterus increased significantly from puberty to postpuberty ($P < 0.05$). TTR expression in the ovary was significantly higher at the period of puberty than prepuberty and postpuberty ($P < 0.05$). The expression of TTR genes in the oviduct was significantly higher at the period of prepuberty and puberty than postpuberty ($P < 0.05$) (Fig. 6). Changes of TTR gene expression in the hypothalamus, pituitary, and uterus at different pubertal stages suggest that TTR genes may be involved in the initiation of puberty in sheep.

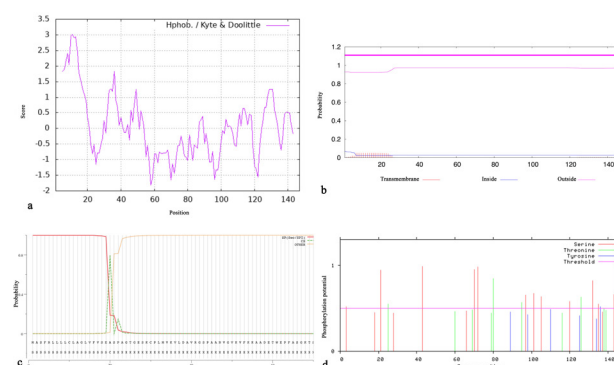


Fig. 4. a: Hydrophobicity analysis of TTR protein in Dolang sheep. b: Prediction of TTR protein transmembrane domain in Dolang sheep. c: Prediction of signal peptide of TTR protein in Dolang sheep. d: Prediction of TTR protein phosphorylation sites in Dolang sheep.

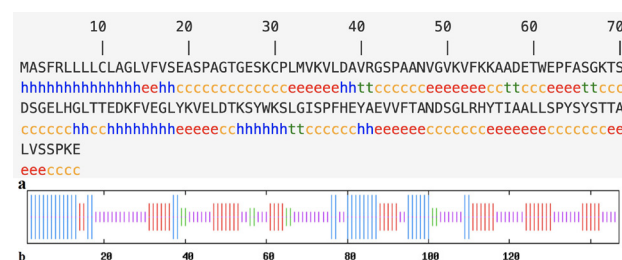


Fig. 5. a: h, Alpha helix; t, Beta turn; c, Random coil; e, Extended strand. b: The lines represent alpha helix, extended strand, beta turn and random coil by the length.

Expression of TTR gene in granulosa cells of the ovaries

The pEGFP-N1-TTR overexpression vector and pEGFP-N1 empty vector were constructed, and the double-digestion identification results showed bright target bands, indicating successful vector construction (Fig. 7). After transferring the TTR gene into ovarian granulosa cells, the fluorescence brightness of the test and control groups at 24 h was examined (Fig. 8), and TTR gene expression was measured at 24 h, 48 h, and 72 h (Fig. 9). Transfection

efficiency was found to be effective, and the expression of the *TTR* gene increased with the increase in transfection time, which proved that the *TTR* gene was successfully transfected into ovarian granulosa cells.

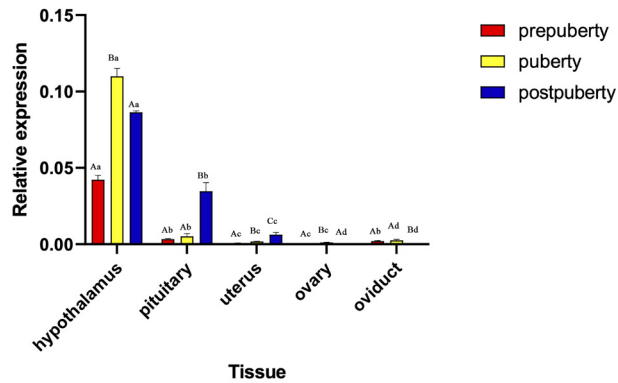


Fig. 6. The relative expression levels of *TTR* gene in different tissues at different puberty stages of Dolang sheep. (1): Values of the same tissues, different periods with different capital letter superscripts indicate significant differences ($P < 0.05$), whereas with the same capital letter or no letter superscripts, there was no significant difference ($P > 0.05$). (2): Values of the same period, different tissues with different small letter superscripts indicate significant difference ($P < 0.05$), while with the same small letter superscripts, there was no significant difference ($P > 0.05$).

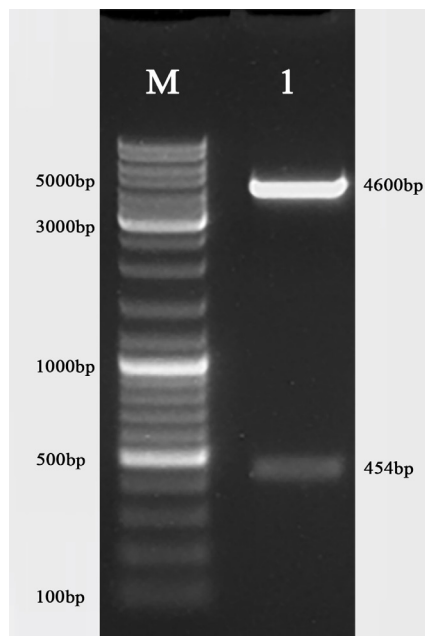


Fig. 7. Identification of recombinant plasmid pEGFP-N1-TTR by double digestion. M:DM10000 DNA maker; 1: Target band.

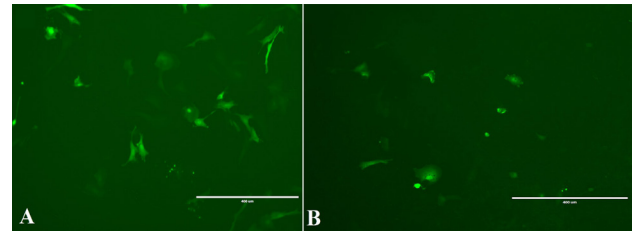


Fig. 8. a: Fluorescence detection graph of control group 24 h after transfection. b: Fluorescence detection graph of 24 test groups after transfection.

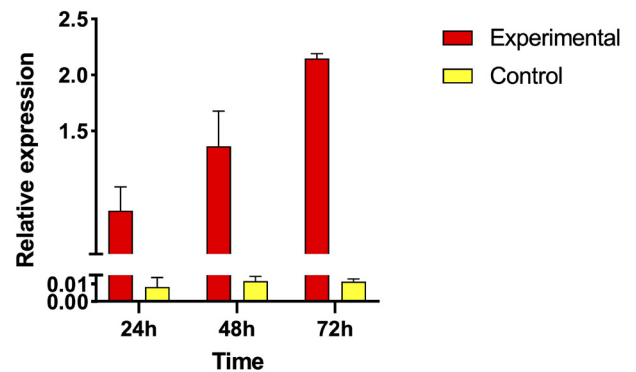


Fig. 9. Changes in *TTR* gene expression at 24 h, 48 h, and 72 h after transfection.

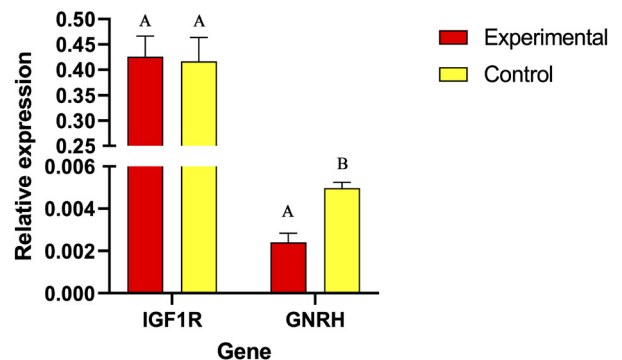


Fig. 10. Effect of *TTR* gene overexpression on the expression of *IGF1R* and *GnRH* genes. Different capital letters indicate significant differences in the experimental and control groups.

Effect of *TTR* genes on *IGF1R* and *GnRH* genes.

IGF1R and *GnRH* genes were examined in ovarian granulosa cells 24 h after transfection, and it was found that there was no significant change ($P > 0.05$) in *IGF1R* gene expression and a significant decrease ($P < 0.05$) in *GnRH* gene expression after the introduction of the *TTR* gene (Fig. 10). Our results suggest that *TTR* may regulate

the initiation of puberty in Dolang sheep by regulating the expression of the *GnRH* gene.

DISCUSSION

TTR is a protein that binds and transports thyroid hormones in the blood together with thyroxine-binding globulin (TBG) and albumin (Zhan *et al.*, 2006). TTR is the major transporter of thyroid hormones in rodents, whereas birds are dominated by TTR and serum albumin (ALB). TTR has been relatively well studied in humans and mice, whereas it has been relatively less studied in sheep.

TTR cDNA sequences have been cloned in more than ten species (Schreiber and Richardson, 1997). It has been shown that *TTR* mRNA transcripts are between 0.65-0.7 kb in length and encode 127-130 amino acids (Kong, 2008). Our result showed *TTR* mRNA transcript are 610bp and encode 147 amino acids in Dolang sheep. The smaller differences in this may be related to the different species studied. Chicken and mammalian *TTR* genes are expressed in both the choroid plexus and the liver (Schreiber and Richardson, 1997). Chicken choroid plexus *TTR* mRNA expression was 47-fold higher than that in the liver (Duan *et al.*, 1991). *TTR* mRNA expression in duck was highest in the choroid plexus, similar to that of chickens, reptiles and mammals (Zhan *et al.*, 2006). In a previous study, it was found that *TTR* gene expression in the hypothalamic tissue was significantly lower in prepubertal and postpubertal Dolang sheep than in puberty (Zhang *et al.*, 2022). In the present study, we reached the same conclusions. *TTR* gene is mainly expressed in the hypothalamus and reaches its highest level in puberty of Dolang sheep. This suggests that *TTR* gene operates mainly in the hypothalamus during pubertal initiation. In addition, small amounts were expressed in the pituitary and uterus and reached a maximum in postpuberty, suggesting that *TTR* gene in pituitary and uterus are also involved in the regulation of puberty.

Additionally, we performed bioinformatic analysis. The results showed that the secondary structure of the TTR protein mainly consisted of an α -helix, extended chain, and irregular coiling, and belonged to an amphipathic acidic protein without a transmembrane structural domain, but contained a signal peptide. It was speculated that the TTR protein was not a membrane protein localized in the biological membrane and underwent translocation after synthesis. Protein phosphorylation is a reversible post-translational modification that can change the conformation of a protein, thereby activating or inactivating it (Zeng *et al.*, 2021). Prediction of TTR protein phosphorylation sites identified 18 phosphorylation sites, including 13 serine (S) sites, four threonine (T) sites, and one complex (Y) site.

It has been shown that the *TTR* gene can drive nuclear translocation of the insulin-like growth factor 1 receptor (IGF-1R) (Vieira *et al.*, 2015), which in turn may allow the function of IGF1 to be affected. IGF1 stimulates the release of GnRH (Hiney *et al.*, 2009), which is involved in the regulation of puberty initiation in sheep (Wojniusz *et al.*, 2011). Therefore, we hypothesized that TTR could regulate pubertal initiation in sheep by inhibiting the release of GnRH. Our results indicate that TTR represses the expression of the *GnRH* gene in Dolang sheep, which is consistent with previous findings. However, no regulatory effect of the *TTR* gene on *IGF1R* gene expression was found, indicating that the *TTR* gene may regulate the IGF1R protein by other means. Our results suggest that TTR may regulate the initiation of puberty in Dolang sheep by regulating the expression of the *GnRH* gene, which provides a new theoretical basis for studying the initiation of puberty in Dolang sheep.

CONCLUSION

In this study, the cDNA sequence of the *TTR* gene 610 bp was successfully cloned from the hypothalamus tissue of Dolang sheep, including 5'UTR 11 bp, 3'UTR 115 bp, and CDS region 444 bp. TTR was mainly expressed in the hypothalamus of Dolang sheep, and its expression increased and then decreased in the hypothalamic tissue during prepuberty, puberty, and postpuberty. TTR has a repressive effect on *GnRH* gene expression and may be involved in the initiation of puberty in Dolang sheep through the regulation of GnRH. Our results have important implications for breeding early pubertal Dolang sheep.

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IRB approval

Ethics Committee of Tarim University of Science and Technology approved the study (Approval Code: 202000301TD, Approval Date: 2020-02-05)

Ethical statement

This study was approved by the Ethics Committee of the Ethics Committee of Tarim University of Science and

Technology.

Institutional review board statement

This study was conducted in accordance with the specifications of the Ethics Committee of Tarim University of Science and Technology.

Data availability statement

Not applicable.

Statement of conflict of interest

The authors have declared no conflict of interest.

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