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Research Article

Sheep *IGFBP2* and *IGFBP4* promoter methylation regulates gene expression and hair follicle development

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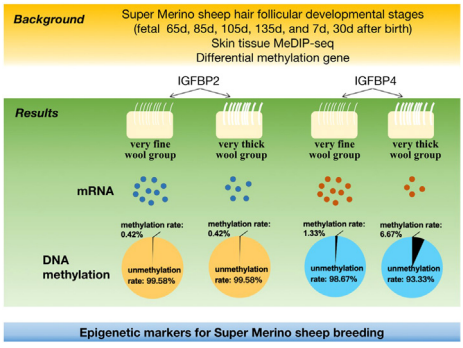
GRAPHICAL ABSTRACT



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ABSTRACT

Background: Hair follicle development is important for the fineness of wool. Our previous methylated DNA immunoprecipitation sequencing showed that *IGFBP2* and *IGFBP4* were differentially methylated at different development stages of the hair follicle of Super Merino sheep. This study selected these two genes as candidates to uncover the influence of their promoter methylation on hair follicle development.

Results: The results showed that the expression levels of *IGFBP2* and *IGFBP4* in the skin of very fine wool Super Merino sheep were higher than that in the very thick wool group, and the difference for *IGFBP4* was significant. The total methylation rate of *IGFBP2* promoter was extremely low, and no significant difference was found in the skin tissue of the Super Merino sheep at different fineness groups. However, the total methylation rate of *IGFBP4* promoter in the very thick group was significantly higher than that in the very fine group, and the total DNA methylation levels of the *IGFBP4* were significantly negatively correlated with mRNA expression. Furthermore, the transcription factor prediction results showed 104 and 91 candidate binding sites for transcription factors in the promoter regions of *IGFBP2* and *IGFBP4*, respectively. Among these, JUN, EP300, SP1, PAX6, ETS1, and MYOD1 may be related to hair follicle development.

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Conclusions: The total methylation level of *IGFBP4* promoter was negatively correlated with its mRNA expression in Super Merino sheep skin tissues with different degrees of fineness, which could be used as an epigenetic marker for wool breeding in Super Merino sheep.

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1. Introduction

Wool is an important economic trait in sheep production, and its quality and yield are determined by many factors, including the morphogenesis and characteristics of the hair follicle [1]. Developing an understanding of the mechanism of hair follicle development is essential for obtaining a general insight into the mechanisms of controlling wool fineness to improve the economic benefits of sheep industry. The Super Merino sheep is a new breed with ultra-fine wool that is independently bred in China. This breed has been jointly bred by several units in Xinjiang, Inner Mongolia, Jilin, and other provinces since 2000 [2,3]. As an ultra-fine wool Merino sheep, the regulation of its hair follicle development and wool fineness is unknown and requires further exploration for better breeding.

In recent years, the study of epigenetics has excited an exceptional degree of attention. Epigenetics is the study of heritable changes in gene expression or cell phenotype through some mechanism without altering the DNA sequence, such as DNA methylation. DNA methylation has been found to have an outsized influence on many processes of biology, including hair follicle development [4,5,6,7,8,9]. Whole-genome bisulfite sequencing (WGBS) conducted on the skin tissue of cashmere goats identified that the methylation status was higher in the skin with hair follicles in the telogen than in the anagen stage [6]. RNA-seq and WGBS performed on the embryonic skin tissues of cashmere goats showed that the DNA methylation in the differentiation stage of the hair follicle was lower than that in the induction stage, and the expression level of certain hair follicle differentiation genes and transcription factor genes was negatively correlated with DNA methylation level [7]. The results suggested that DNA methylation may play a role in hair morphogenesis by regulating gene expression [6,7]. Methylation levels in the promoters of several genes have been identified as correlating significantly with gene expression levels and hair follicle development, such as in BMP4 [10], HOXC8 [11], and H19 [12].

Recently, our group analyzed the DNA methylome and the whole transcriptome in the skin tissue of 18 Super Merino sheep at six developmental stages of the hair follicle (fetal at 65 d, 85 d, 105 d, and 135 d and at 7 d and 30 d after birth). The results showed that the methylation levels upstream of the transcription initiation site were lower in the later stages than in the earlier stages [13]. The differential methylation genes were enriched in Wnt, TNF, TGF-beta, and other signaling pathways related to hair follicle development [14]. Furthermore, the methylation of *IGFBP2* and *IGFBP4* was different in the skin tissues at different developmental stages of Super Merino sheep [14]. *IGFBP2* and *IGFBP4* are important members of the insulin-like growth factor binding protein (IGFBP) family, which is closely involved in hair follicle development [15,16]. *IGFBP2* and *IGFBP4* can interact with other binding proteins to affect cell proliferation and the development of hair follicles [15,17,18,19]. Hence, this study analyzed the influence of *IGFBP2* and *IGFBP4* promoter methylation on mRNA expression levels of *IGFBP2* and *IGFBP4* in skin tissues of Super Merino sheep of different fineness of wool and predicted the binding sites of transcription factors in the promoter regions of *IGFBP2* and *IGFBP4*. The results of this study will provide a theoretical basis for the understanding of the regulatory mechanism of hair follicle development.

2. Materials and methods

2.1. Sample collection

The experimental process was consistent with protocols of international guides for the ethical use of animals in research. The experimental sheep were 1-year-old Super Merino ewes from Gongnaisi Breeding Farm in Yili, Xinjiang, which were all at the same feeding and management. The sheep were divided into a very fine group (16.90 μm) and a very thick group (19.53 μm), with five sheep samples in each group. Skin tissue of the left scapula was collected and stored in a cryopreserved tube in −80°C refrigerator for subsequent analysis.

2.2. DNA and RNA extraction and primer design

About 50–100 mg tissue samples were triturated in liquid nitrogen, and DNA and RNA were extracted from the skin tissues of 10 sheep (five very fine group sheep and five very thick group sheep). Tissue genomic DNA extraction kit (Tiangen, Beijing, China) was used to isolate DNA [2]. The extracted genomic DNA was subjected to a Nanodrop One spectrophotometer and Qubit 2.0 electrophoresis for qualified quality inspection and further experimentation. Tissue RNA was extracted by the AllPrep DNA/RNA Mini Kit (Tiangen, Beijing, China), and RNA concentration was determined using Nanodrop 2000 nucleic acid detector. After agarose gel electrophoresis, cDNA was synthesized using a reverse transcription kit (PrimeScript RT reagent Kit with gDNA Eraser, TaKaRa, Dalian, China) and stored for a further experiment at the −20°C [2]. Primer Premier 5.0 software was used to design the primers synthesized by Shanghai Sangon Biotech Co., Ltd (Shanghai, China). The primer sequences used for the *real-time quantitative reverse transcription polymerase chain* (qRT-PCR) and bisulfite sequencing PCR (BSP) study in this experiment are shown in Table 1.

2.3. The qRT-PCR reaction

The expression levels of *IGFBP2* and *IGFBP4* were detected using qRT-PCR. *GAPDH* was used as internal control, and the corresponding *IGFBP2* and *IGFBP4* primers were used to make three parallel samples for amplification. The reaction procedure for qRT-PCR included pre-denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 62.8°C for 30 s, and extension at 95°C for 5 s.

2.4. Bisulfite sequencing PCR amplification

The steps of the DNA modification were performed according to the instructions of the EZ DNA Methylation-Gold Kit (Zymore-

Table 1
Primers used in this study.

Gene	Primer sequence (5′→3′)	Product size (bp)	Annealing temperature (°C)
qPCR-IGFBP2	F: GGTTGCAGACAATGGCGAGGAG R: TGCTGCTCCGTGACCTTCTCC	162	60
qPCR-IGFBP4	F: AGAGCCGCACCCACGAAGAC R: GTCCACACACCAGCACTTGCC	125	60
qPCR-GAPDH	F: GAGATCAAGAAGGTGGTGAAGCAG R: GTAGAAGAGTGAGTGTCGCTGTTG	113	60
BSP-IGFBP2	F: TGAGTTATTGAAATTTAAGTTAGGT R: CATTCTATAAAACAAAAAAAACTCC	202	48.6
BSP-IGFBP4	F: GGGTTAAGATAGATGGGGTAGAAG R: AAACAAAACCAAAAATTACAAACTAAC	147	48.6

Note: qRT-PCR, real-time quantitative reverse transcription polymerase chain; BSP, bisulfite sequencing PCR.

search, USA). The modified DNA was stored in a −80°C refrigerator for subsequent experiments. The PCR program used for BSP included pre-denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 45 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, ending at 72°C for 10 min. PCR amplification product was purified with a PCR product purification kit (Tiangen, Beijing, China). After agarose gel electrophoresis and concentration determination, the purified product was linked to a PMD19-T vector (TaKaRa, Dalian, China) [20]. Three positive clones were selected from each sample and sent to Sangon Biotech (Shanghai, China) for sequencing.

2.5. Bioinformatic analysis to predict transcription factors

The promoter sequences of sheep *IGFBP2* and *IGFBP4* were downloaded from the NCBI database. The transcription factors in the promoter regions of *IGFBP2* and *IGFBP4* were predicted using PROMO, UCSC, and GeneCards database. Metascape was used for the enrichment analysis of the predicted transcription factors. To determine the relationship between enrichment terms, the Kappa score was calculated as a measure of similarity between terms. The similarity network of enrichment terms was constructed using the STRING database to analyze the protein–protein interaction network of transcription factors, and the key transcription factors were selected. ORF Finder software was used to predict the ORF of transcription factors. The online softwares ProtParam, SOPMA, and SWISS-MODEL were used to predict the primary, secondary, and tertiary structures of transcription factors.

2.6. Statistical analysis

SPSS19.0 was used to analyze the differences in expression for *IGFBP2* and *IGFBP4*. The average value of the data from three repeated tests was used for each object of analysis. Relative quantitative analysis was performed using the $2^{-\triangle\triangle Ct}$ formula [21]. SPSS software was utilized to analyze the correlations between methylation levels and mRNA expression. The data were presented as means ± SEMs.

3. Results

3.1. Expression analysis of IGFBP2 and IGFBP4 in skin tissues with different fineness

The amplification curves and fusion curves of *IGFBP2* and *IGFBP4* and the reference gene GAPDH showed good coincidence and had a shared single peak. The results of the qRT-PCR indicated that the expression level of *IGFBP2* in the skin tissue of very fine wool Super Merino sheep was higher than that in the very thick wool skin group, but the difference was not significant ($P > 0.05$, Fig. 1). However, the expression level of *IGFBP4* in the skin tissue of very fine wool Super Merino sheep was significantly higher than that in the very thick group ($P < 0.01$, Fig. 1).

3.2. CpG site prediction, product detection, and sequence alignment

The gene sequences for *IGFBP2* and *IGFBP4* were copied into the MethPrimer to provide an analysis of the CpG site of the target DNA sequence. As shown in Fig. S1, both *IGFBP2* and *IGFBP4* promoter regions have a CpG island. The online software MethPrimer was used to design the methylation primers within or next to the CpG island of the sheep *IGFBP2* and *IGFBP4* (Table 1). The methylation primers for *IGFBP2* and *IGFBP4* contain 16 and 5 methylation sites, respectively (Fig. S1 and Fig. S2). DNA methylation analysis was carried out on the promoter regions of *IGFBP2* and *IGFBP4* in Super Merino sheep, and the methylation patterns for the skin tissues of Super Merino sheep of the two fineness groups were analyzed (the very fine group and the very thick group). Gel electrophoresis of the PCR products of the *IGFBP2* and *IGFBP4* methylation primers showed the correct size of the PCR product, which indicates that the PCR product could be used for subsequent experiments (Fig. 2). The sequencing peak map for the methylation regions of *IGFBP2* and *IGFBP4* in Super Merino sheep showed 16 and 5 methylation sites in the analyzed region, respectively, which was consistent with the prediction (Fig. 3).

3.3. Analysis of DNA methylation patterns and correlation analysis between methylation and mRNA

The promoter methylation levels of *IGFBP2* and *IGFBP4* in Super Merino sheep were analyzed (five sheep in very fine group and five sheep in very thick group). In *IGFBP2*, the methylation level of CpG island was extremely low in the very fine group and the very thick group, and the methylation rate of CpG island was 0.42% in both groups. The methylation rate of CpG15 in the very fine group (6.67%) was higher than that in the very thick group (0%), and the methylation rate of CpG2 in the very fine group (0%) was lower than that in the very thick group (6.67%) (Fig. 4). The DNA methylation level of *IGFBP2* was the same between the two groups, preventing correlation analysis from being performed. For *IGFBP4*, the methylation level of the CpG island was low in the very fine group and the very thick group, and the methylation rates for the sites CpG1, CpG2, and CpG3 in the very fine group were lower than the rates in the very thick group (Fig. 4). In addition, the DNA methylation levels of *IGFBP4* were significantly negatively correlated with mRNA expression ($P < 0.01$).

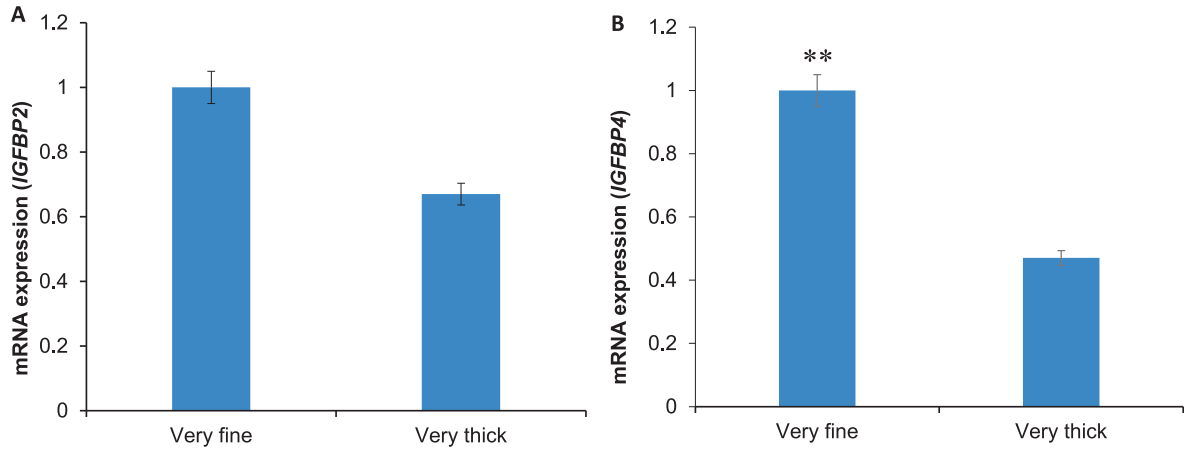

Fig. 1. The mRNA expression of *IGFBP2* (A) and *IGFBP4* (B) in different fineness groups of skin tissues ** represents very significant difference ($P < 0.01$).

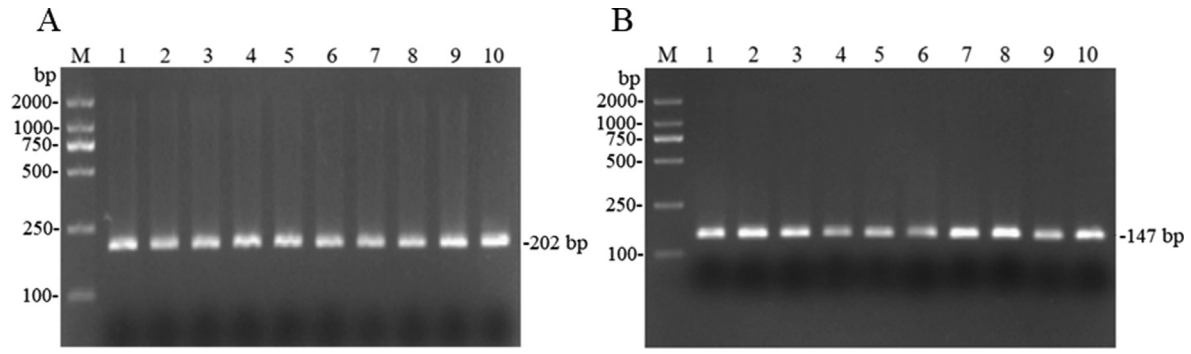

Fig. 2. PCR product gel electrophoresis of *IGFBP2* (A) and *IGFBP4* (B) in Super Merino sheep. M is DL2000 marker, and lines 1–10 are PCR products.

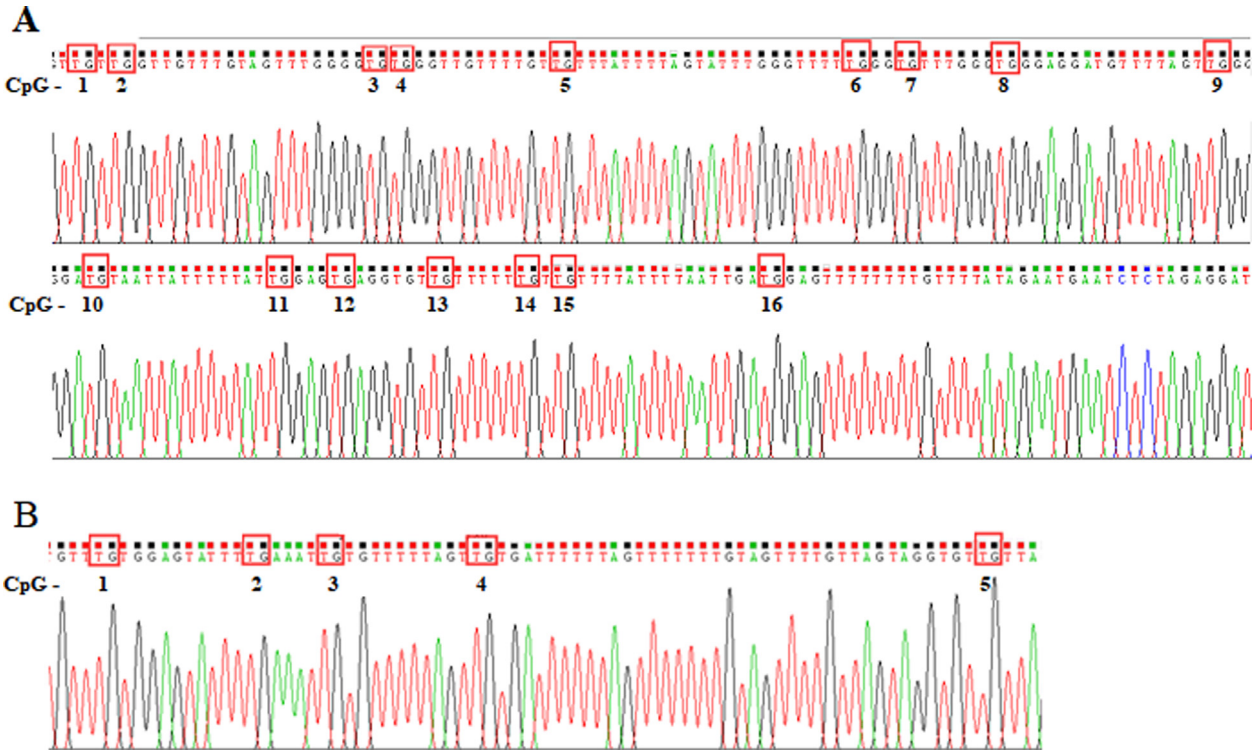

Fig. 3. Sequencing map of methylation regions of *IGFBP2* (A) and *IGFBP4* (B) gene in Super Merino sheep. Boxes and the numbers indicate CpG sites.

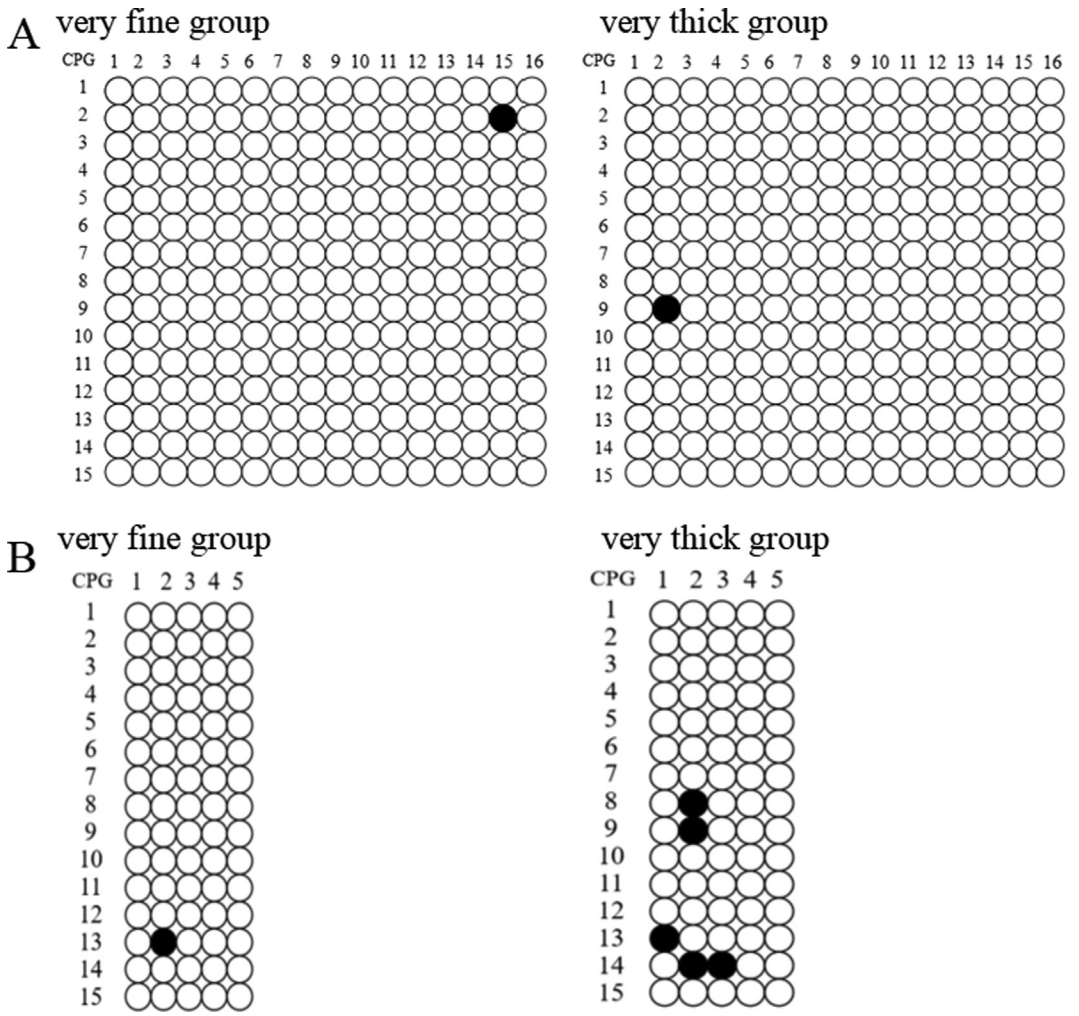

Fig. 4. Methylation pattern of CpG loci of *IGFBP2* (A) and *IGFBP4* (B) genes in Super Merino sheep. ●, methylated; ○, unmethylated.

3.4. Prediction of transcription factors in the promoter regions of IGFBP2 and IGFBP4 and GO enrichment analysis

Using PROMO, UCSC, and GeneCards, 104 and 91 transcription factors were predicted to bind to the promoter regions of *IGFBP2* and *IGFBP4* (Table S1). The enrichment analysis of the transcription factors for *IGFBP2* and *IGFBP4* using MetaScape showed that the transcription factors enriched in the promoter of *IGFBP2* were focused on the following aspects: response of gland development cells to hormone stimulation, negative regulation of embryonic organ development cell proliferation, regulation of neuronal death, development of the sensory organs, epithelial cell proliferation, regulation of DNA binding to multicellular organism growth, and reproductive structure development, and so forth (Fig. 5). The transcription factors enriched in the promoter of *IGFBP4* were focused on the following aspects: gland development, chordate animal embryo development, liver development, AP1 pathway sensory organ development, epithelial cell proliferation, regulation of epithelial cell differentiation, and so on (Fig. 5). These results indicate the multifunction of *IGFBP2* and *IGFBP4*.

3.5. Network analysis of potential transcription factors

To further analyze the relationships between these factors, all transcription factors were input into the STRING database to obtain the PPI network according to their interaction. The binding protein interaction network of common key transcription factors was screened out. The key transcription factors included JUN, EP300, SP1, PAX6, ETS1, and MYOD1 (Fig. 6).

3.6. Results of transcription factor structure prediction

ProtParam online software was used to predict the primary structure of JUN, EP300, SP1, PAX6, ETS1, and MYOD1 transcription factors in sheep (Table S2). The prediction results for the secondary structure are shown in Fig. S3. The main elements of the secondary structure of the proteins encoded by the six transcription factors were all random coils. The prediction results for the tertiary structure of the protein encoded by sheep JUN, EP300, SP1, PAX6, ETS1, and MYOD1 transcription factors are shown in Fig. 7. JUN was mainly composed of an α helical elongated chain. The EP300, SP1, PAX6, ETS1, and MYOD1 were mainly composed of an α helical elongated chain. The prediction results of the tertiary structure of the proteins encoded by the above six transcription factors by the random curling of the β corner were generally the same as those obtained for the secondary structure (Fig. 7).

4. Discussion

The quality and fineness of wool are determined by the structure and characteristics of the hair follicles, so studying the mechanism of regulation of the structure of the hair follicle and its

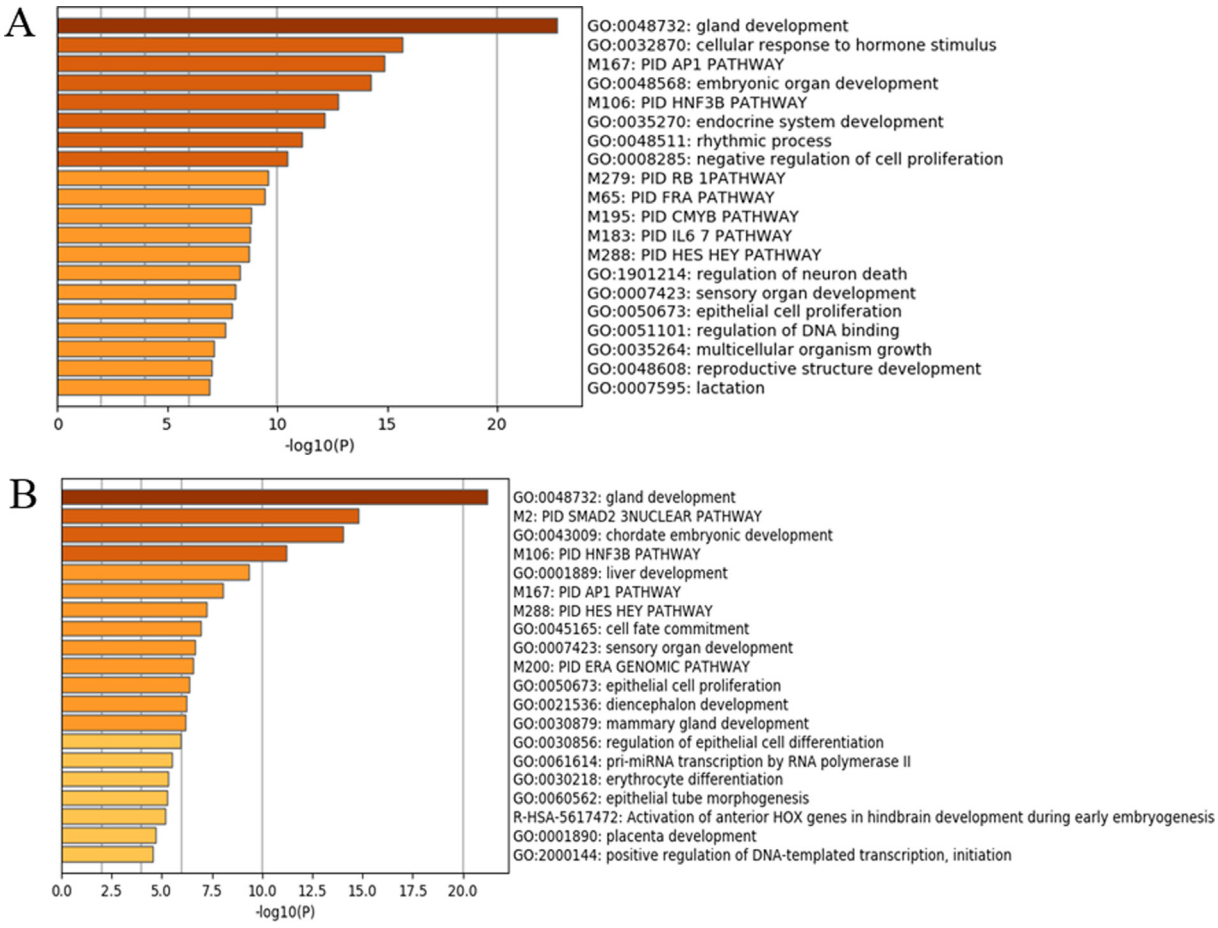

Fig. 5. GO enrichment analysis of transcription factors of *IGFBP2* (A) and *IGFBP4* (B).

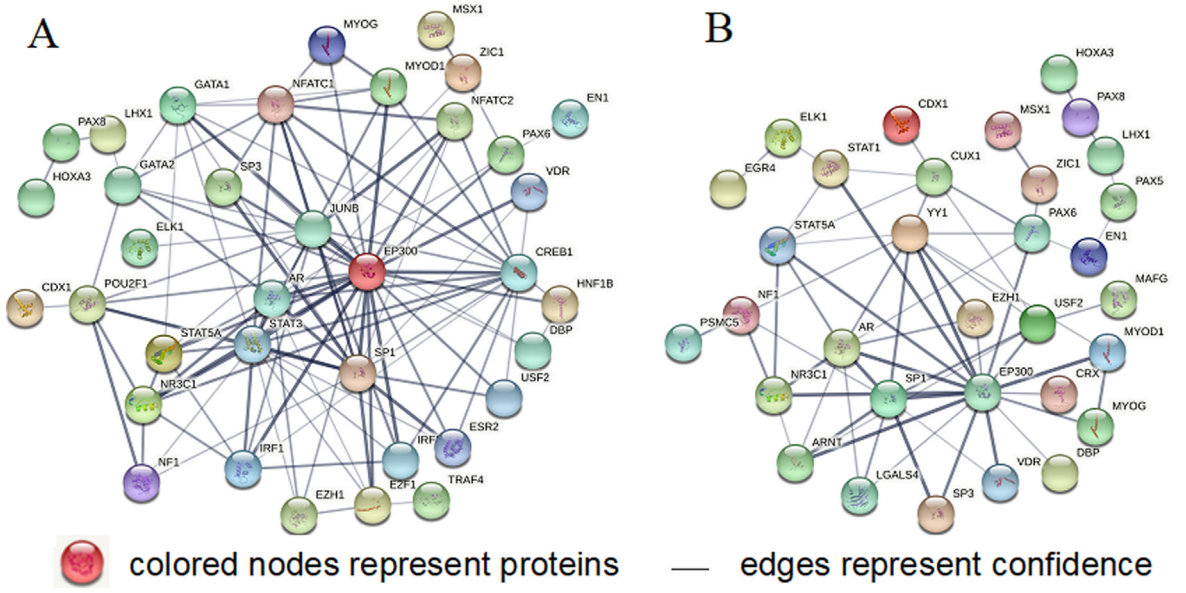

Fig. 6. Protein interaction network of transcription factors of *IGFBP2* (A) and *IGFBP4* (B). Edges represent protein–protein associations; the thickness of the edge represents edge confidence (thick edges represent high levels of confidence).

development is of great importance. More and more functional molecules have been identified and characterized in each stage of the hair follicle development of mice [22,23]. However, few reports have been published regarding the mechanism that underlies the morphogenesis of fine sheep hair follicles due to the associated technical difficulties and high cost. There are conservative signals

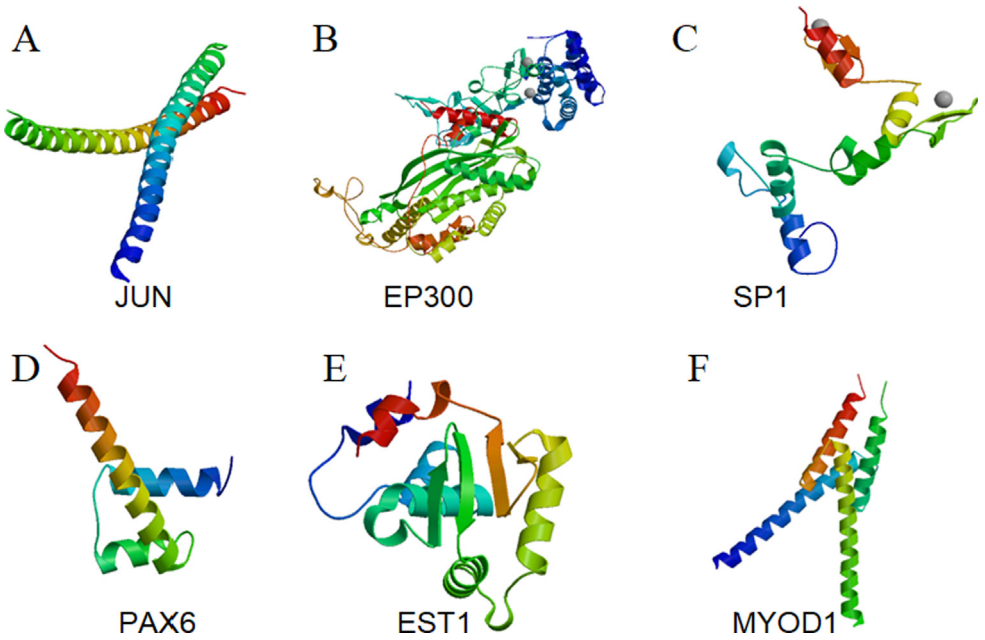

Fig. 7. Prediction of tertiary structure of transcription factors.

in hair follicle development in mice, but physiology and regulation mechanisms are different between mice and sheep. There have been few studies on epigenetic markers and functional mechanisms related to hair follicle development in sheep. Further exploration and integration of the results of epigenetic studies are needed to better understand the mechanisms of the regulation of sheep hair follicle development.

In Super Merino sheep, MeDIP-seq identified some differential methylation genes, including *IGFBP2* and *IGFBP4*. *IGFBP2* and *IGFBP4* are members of the IGFBP family, which has an important influence on hair follicle development [15,16]. Studies have also shown that gene promoter methylation can inhibit the binding of transcription factors in the promoter region, leading to a decline in the gene transcription level or even the cessation of transcription, achieving the purpose of regulating gene transcription [6,7,8]. Therefore, we detected the methylation level and expression level of *IGFBP2* and *IGFBP4* in the skin tissues of sheep with different wool fineness. The expression level of *IGFBP4* in the skin tissue of the very fine Super Merino sheep was significantly higher than that in the very thick group, and the DNA methylation level of *IGFBP4* was significantly negatively correlated with mRNA expression. These results suggest that the expression and function of *IGFBP4* with respect to hair follicle development may be regulated by its promoter methylation.

Unfortunately, the specific mechanisms through which *IGFBP4* regulates hair follicle development have not been reported. IGFBP4 features the main functional protein domains (IGFBP domain and thyroglobulin type-I domain), similar to other genes in the IGFBP family. Thus, we can speculate on the mechanisms by which *IGFBP4* regulated hair follicle development based on relevant studies. IGFBPs are important proteins that interact with IGFs, and IGFs have a regulatory function in hair follicle development [24,25]. Furthermore, the functions of some genes in the IGFBP family on hair follicle development have been identified. IGFBP5 is identified as the first molecular marker among different types of hair follicle, and it can regulate hair shaft differentiation and hair type determination [26,27]. The activation of Sonic hedgehog (Shh) pathway can stimulate the regeneration of dermal papilla [28], and IGFBP2 can interact with the Shh pathway in the hair follicle [17]. IGFBP1 also has a positive effect on hair growth [29]. Based on this, we speculated that the functional mechanism of IGFBP4 on hair follicle development may be the same as that of the other IGFBP genes, but the actual function requires further experimental verification.

Many studies have shown that gene promoter methylation can inhibit the binding of transcription factors in the promoter region, and this leads to a decline in gene transcription level or even the cessation of transcription. Hence, we analyzed candidate transcription factors that can bind to the promoter region of *IGFBP2* and *IGFBP4* to explore whether these methylations of *IGFBP2* and *IGFBP4* affect the binding of transcription factors. Many important transcription factors have been identified, such as C/EBPs, PPARγ, ADD1, PDX1, NGN3, and FOSL2. Some of these transcription factors have been identified to have close relationship with hair follicle development [30]. The enrichment analysis of the transcription factors of *IGFBP2* and *IGFBP4* using MetaScape showed that the binding transcription factor of *IGFBP2* focused on the response of gland development cells to hormone stimulation, the negative regulation of embryonic organ development cell proliferation, regulation of neuronal death, sensory organ development, epithelial cell proliferation, and so on [31]. The binding transcription factor of *IGFBP4* has functions that are focused on gland development, chordate animal embryo development, epithelial cell proliferation, regulation of epithelial cell differentiation, and so forth. Some of these functions play a crucial role in hair follicle development, which may provide a theoretical basis for identifying more transcription factors related to hair follicle development.

In the protein interaction network, six common key transcription factors were screened out, namely, JUN, EP300, SP1, PAX6, ETS1, and MYOD1. These transcription factors can regulate the cell cycle, cell proliferation, gene transcription, glucose uptake, and growth and development [32,33,34]. Meanwhile, the secondary and tertiary structure of the transcription factors for JUN, EP300, SP1, PAX6, ETS1, and MYOD1 have been predicted, which would lay the foundation for a subsequent verification experiment. However, the mechanism of how the promoter regions of *IGFBP2* and *IGFBP4* genes regulate hair follicle development and wool growth remains further verified. In the future, the effect of these transcription factors on hair follicle development could be further studied.

5. Conclusions

The promoters of *IGFBP2* and *IGFBP4* were hypomethylated in the skin tissue of Super Merino sheep. The DNA methylation of *IGFBP4* was negatively correlated with its expression level, which indicates that DNA methylation was negatively regulated with gene expression level. In addition, transcription factors, including JUN, EP300, SP1, PAX6, ETS1, and MYOD1, were predicted to bind to the promoter regions of *IGFBP2* and *IGFBP4*. The results of these studies could be used to provide epigenetic markers for Super Merino sheep and provide the theoretical basis for sheep wool breeding.

Author contributions

Study conception and design: K Tian, X Huang, Y Tian.
-Data collection: K Tian, X Huang, X Xu, W Wu, J Di.
-Analysis and interpretation of results: J He, B Zhao, X Fu.
-Draft manuscript preparation: Y Tian, J Du, X Yang, W Zeng.
-All authors reviewed the results and approved the final version of the manuscript.

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Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

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