Skin Transcriptome Analysis Identifies the Key Genes Underlying Fur Development in Chinese Tan Sheep in the Birth and Er-mao Periods

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Abstract

Background

Hair follicle development in Tan sheep differs significantly between the birth and Er-mao periods, but the underlying molecular mechanism is still unclear.

Methods

We profiled the skin transcriptomes of Tan sheep in the birth and Er-mao periods via RNA-seq technology. The Tan sheep examined consisted of three sheep in the birth period and three sheep in the Er-mao period.

Results

A total of 364 differentially expressed genes (DEGs) in the skin of Tan sheep between the birth period and the Er-mao period were identified, among which 168 were upregulated and 196 were downregulated. Interestingly, the *FOS* proto-oncogene (*FOS*) (**fold change=22.67, P value=2.15*10⁻⁴⁴**) was the most significantly differentially expressed gene. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis found that the *FOS* gene was significantly enriched in the signaling pathway related to hair follicle development. Immunohistochemical analysis showed that the *FOS* gene was expressed in the skin of Chinese Tan sheep at the birth and Er-mao periods, with abnormally high expression in the Er-mao period.

Conclusions

Our findings suggest that the *FOS* gene promotes hair follicle development in Tan sheep.

Background

The Chinese Tan sheep, which is mainly distributed in Ningxia Province and adjacent areas, is one of the most important sheep breeds used for the production of high-quality fur [1] and famous worldwide for its meat, wool, and fur. Tan sheep fur, commonly known as Er-mao fur, is from the pelt of the lamb approximately 35 d after birth (which is traditionally called the age at Er-mao) [2]. At this time, the fur is thin and soft with good thermal performance, and its wool is curved like waves; Chinese Tan sheep wool is thus said to “nine bay”.

Tan sheep hair development and growth cannot be studied independent of the hair follicles. The hair follicle is a small, dynamic organ that undergoes general and periodic changes throughout its lifespan [3]. The villi of mammals are made up of hair follicles, which make up the mammalian coat. Hair follicles are classified as primary follicles or secondary follicles. Primary follicles are larger in diameter and have a complete sebaceous gland accessory structure. Secondary follicles are smaller in diameter and more numerous than primary follicles and lack a complete sebaceous gland accessory structure. In the process of hair follicle development in the Chinese Tan sheep, at 90 d, the Tan sheep embryo has formed primary hair follicles, and hair fibers have grown out of the skin. The 120 d Tan sheep embryo exhibits a mixed

distribution of primary hair follicles and secondary hair follicles all over its body. Tan sheep fur traits facilitate the performance of the Tan sheep in a critical period of growth and development (from birth to the Er-mao period, approximately 30 days). Fur formation between the birth and Er-mao periods may be related to development of the hair follicles or regulation of the expression of some genes.

Therefore, to comprehensively investigate the intrinsic molecular mechanisms of hair follicle development in Tan sheep between the birth and Er-mao periods, we performed RNA-seq analysis of skin tissues from six Tan sheep: three sheep in the birth period and three sheep in the Er-mao period. To identify key factors related to hair follicle development in Tan sheep in the birth and Er-mao periods, we combined multiple methods: principal component analysis (PCA), differential expression (DE) analysis, and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses. Moreover, to validate potential genes related to hair follicle development identified in our study, we performed immunohistochemical analyses of skin tissues from Tan sheep in the birth and Er-mao periods. This study will provide a reference for the study of the internal molecular mechanism of hair follicle development in Chinese Tan sheep.

Results

Morphological observation of Tan sheep skin at the two time periods

The fur of the Chinese Tan sheep in the birth and Er-mao periods differed. Reports have shown that Tan sheep fur has a high growth rate from birth to the Er-mao period [4]. In the birth period, the wool of the Chinese Tan sheep contained different types of hair. The wool was short, with a length of approximately 4.5 cm (Fig. 1A). In the Er-mao period, the villus grew out of the skin, and the coat was composed of the villus and different types of hair. The wool length was approximately 7 cm, and the hair strands curled more than seven times (Fig. 1B). At this point, the two types of coats formed a different spiral structure in particular proportions with the appropriate fineness and length.

Hair follicles also differed between Tan sheep in the birth and Er-mao periods. Compared with those at birth, Tan sheep in the Er-mao period showed a 50% increase in PF diameter and a 34% increase in SF diameter (Fig. 1C, D, E). However, the Tan sheep at the Er-mao period showed a 36% decrease in the number of PFs per unit area and a 54% decrease in the number of SFs per unit area (Fig. 1F).

DE in the skin of Tan sheep in the birth and Er-mao periods

To obtain a comprehensive view of transcriptional changes during hair follicle development in Tan sheep between the birth and Er-mao periods, we performed RNA-seq analysis of three skin samples from Tan sheep from each group.

After quality control, the clean data ratio for all samples was greater than 95%, and the mapping rate for each sample was more than 80% (**Table S2**), which was sufficient for subsequent analysis. PCA by using the FPKM (Fragments per kilobase of transcript per million fragments mapped) values (Fig. 2A) showed that samples from Tan sheep in the birth period differed from those from Tan sheep in the Er-mao period by PC1, and both PC1 and PC2 accounted for 92% of the variation in these samples. The relatively close distance between the two groups is consistent with the short duration between birth and the Er-mao period (only 30 days), indicating similar background gene expression at these two time periods.

Skin transcriptome analysis identified a total of 18607 expressed transcripts in the birth and Er-mao periods in Tan sheep. We found 364 DEGs by using the criteria of a fold change > 2.0 or < -2.0 and a P value < 0.05, with 168 genes upregulated and 196 genes downregulated. Interestingly, the *FOS* gene (**fold change = 22.67, P value = 2.15×10^{-44}**) (Fig. 2B), which was up-regulated, was the most significantly differentially expressed gene. The *FOS* gene, whose full name is *FOS* proto-oncogene, is also named *c-fos*. An immediate-early gene, the *FOS* gene has been shown to play a key role in cell growth, proliferation and differentiation[5–7]

Kegg Enrichment Analysis

A total of seventeen biological pathways were significantly enriched in the DEGs (**Table S4**), and further analysis revealed that the *FOS* gene was significantly enriched in the pertussis, leishmaniasis, Chagas disease (American trypanosomiasis), herpes simplex infection, and MAPK signaling pathways. Furthermore, the MAPK signaling pathway (Fig. 2C) (**Table S4**) was reported to be closely related to hair follicle development [8–10].

Real-time PCR validation of differential gene expression in Tan sheep in the birth and Er-mao periods

The DEGs screened from the RNA-seq data were verified by real-time PCR. The results of validation of the expression of key genes, including *FOS*, showed that (Fig. 2D) the expression patterns of these key genes were consistent with the expression patterns revealed by RNA-seq, indicating that the RNA-seq results were reliable.

Immunohistochemical staining analysis of Tan sheep in the birth and Er-mao periods

To further verify expression of the *FOS* gene in the skin of Tan sheep in the birth and Er-mao periods, we performed immunohistochemical staining of the skin tissues taken from Tan sheep in the birth and Er-mao periods. The immunohistochemistry results and results of statistical analysis of the

immunohistochemistry images showed that the *FOS* gene was abnormally highly expressed in the skin of Tan sheep in the Er-mao period (Fig. 3).

Discussion

The fur produced by the Chinese Tan sheep, a unique sheep breed in China, in the Er-mao period has high economic value. The difference in hair follicle development in Chinese Tan sheep between birth and the Er-mao period leads to differences in coat between these periods. To demonstrate the potential molecular mechanisms that influence this difference in hair follicle development in Tan sheep between birth and the Er-mao period, we analyzed the skin transcriptomes of Tan sheep at these periods.

The use of sufficient biological replicates is essential for any experiment, with the main purpose of biological replicates to eliminate the effects of differences between individuals on the experimental results [11]. In this study, we selected three representative Tan sheep in the birth and Er-mao periods for transcriptome sequencing to ensure that the sequenced data were statistically significant and to eliminate as much as interference from individual differences on the overall experimental results as possible. To ensure the depth and quality of the sequencing data, the data volume for each sample during sequencing was greater than 10 GB, and the clean ratio for each sample after quality control of the raw data was greater than 95%. Meanwhile, the mapping rate for each sample was greater than 80% (**Table S2**). The results of PCA indicated the similar expression of background genes in the birth and Er-mao periods (Fig. 2A).

qPCR (Real-time quantitative PCR) is a type of reverse transcription PCR that requires little RNA, is less laborious than other techniques, and produces a large amount of data over a short time. Its ease of use and sensitivity make it a valuable tool for bioinformatics, virology, and molecular diagnosis, and qPCR is widely regarded as the gold standard for gene expression analysis [12–14]. In recent years, qPCR has become the preferred method to verify the results of gene expression profiling [15]. In this study, the qPCR results showed that the expression patterns of key genes were consistent with those determined by RNA-seq (Fig. 2D), indicating that the RNA-seq results were reliable. In summary, the RNA-seq results were sufficient for subsequent analysis.

In this study, the *FOS* gene, which was upregulated, was found to be the most significantly expressed among all the identified DEGs (Fig. 2B) (**Table S3**). The *FOS* gene is a typical early cellular response gene, and the protein encoded by the *FOS* gene is a typical immediate-early protein in the cell [16–21]. *FOS* is the main nuclear target in the signal transduction pathway involved in the regulation of cell growth, differentiation, and transformation [22]. Studies have shown that the *FOS* gene participates in not only the normal growth, differentiation and apoptosis of cells but also intracellular information transfer and cellular energy metabolism; as such, *FOS* plays a basic and important role in biological activities [23, 24]. It was reported that the *c-fos* gene plays a key role in the proliferation and differentiation of mature cells and the physiological response to the environment [23].

In this study, the qPCR results showed that the *FOS* gene was expressed in the skin of Chinese Tan sheep in the birth and Er-mao periods, with its expression in Chinese Tan sheep higher in the Er-mao period than in the birth period. The results also proved that the *FOS* gene was differentially expressed and upregulated. KEGG pathway enrichment analysis showed that the *FOS* gene was significantly enriched in the signaling pathway related to hair follicle development (Fig. 2C) (**Table S4**), which suggests that the *FOS* gene is closely related to the development of hair follicles in the Chinese Tan sheep. The hair of the Chinese Tan sheep grows gradually from birth to the Er-mao period. The growth of wool depends on the growth and development of hair follicles. HE staining showed that the diameter of the hair follicles was increased, while the number of hair follicles per unit area was decreased in Tan sheep in the birth period compared to those in the Er-mao period (Fig. 1), which indicated that the hair follicles of the Tan sheep gradually developed into their complete form from birth through the Er-mao period. We speculated that the *FOS* gene promotes hair follicle growth and development in Chinese Tan sheep.

To verify expression of the *FOS* gene in the skin tissues of Chinese Tan sheep during the birth and Er-mao periods, immunohistochemical staining was performed. The results showed that the *FOS* gene was expressed in the skin of Chinese Tan sheep during the birth and Er-mao periods, with abnormally high expression in the skin of the Chinese Tan sheep in the Er-mao period (Fig. 3). This result demonstrates that the expression level of the *FOS* gene in skin tissue from Chinese Tan sheep gradually increased from the birth period to the Er-mao period; that is, as the expression level of the *FOS* gene in the skin tissue of Tan sheep gradually increased, the hair follicles of Tan sheep gradually and completely developed. This finding also indicated that the expression of the *FOS* gene is closely related to hair follicle development in Tan sheep and further verifies that the *FOS* gene promotes hair follicle development.

Conclusion

In summary, we report that hair follicle development in Tan sheep from the birth period to the Er-mao period may be regulated by the *FOS* gene. Our findings suggest that the *FOS* gene promotes hair follicle development in Tan sheep. The results of this study provide a reference for research on the internal molecular mechanism of hair follicle development in Tan sheep.

Methods

Animals collection and preparation

The experimental samples were collected from the Ningxia Yanchi Tan sheep breeding farm. Six unrelated Chinese Tan sheep (with no common grandparents) at two different physiological periods (the birth period and the Er-mao period) were selected and divided into the birth (**BS**) and Er-mao (**ES**) groups.

Tissue Samples

For each Tan sheep, after its hair was cut and the iodine was removed with alcohol, approximately 1 cm² of skin tissue was taken with sterile forceps, and the tip of the sample was quickly cut with a sterile scalpel. After the procedures, Yunnan Baiyao (Yunnan Baiyao Group Co., Ltd., China) was used promptly to stop the Tan sheep bleeding. Half of each piece of Tan sheep skin tissue was stored in liquid nitrogen for RNA extraction, and the remaining half was stored in a 4% paraformaldehyde fixing solution for paraffin sectioning. All procedures including handling the Tan sheep samples were approved and implemented by the Laboratory Animal Care and Use Guide jointly developed by the Ministry of Agriculture of the People's Republic of China and the Institute of Animal Science of the Chinese Academy of Agricultural Sciences.

He Staining

Skin tissue samples were cut into 1 cm*0.6 cm*0.6 cm pieces, washed under running water for 24 h, and dehydrated with different concentrations of alcohol. Then, the skin tissue was embedded in EPON 812 with an embedding machine and sliced continuously (5 microns) under an LKB 8800 ultratome to make paraffin sections, which were then stained with hematoxylin and eosin.

The skin tissues of three Tan sheep at both periods were selected for HE staining. Five different visual fields (100×) of each slice were randomly selected. The diameter of primary hair follicles and secondary hair follicles from the skin of Chinese Tan sheep in the birth and Er-mao periods were measured by ImageJ software. Specifically, the diameters of three primary hair follicles and five secondary hair follicles from sheep at each period were counted randomly, and the results were calculated as the mean ± standard deviation. Statistical analysis was carried out by R package, and one-way analysis of variance was used to analyze the differences in primary hair follicle and secondary hair follicle diameter at different time periods.

Rna Isolation And Sequencing

Six individual Tan sheep with complete skin tissue were selected from the ES (birth) and BS (Er-mao period) groups for whole-transcriptome sequencing (RNA-seq) analysis (N = 6 tissue samples). Total RNA was extracted from these 6 samples using the RNeasy Mini Kit (Qiagen, Germany) according to the reagent specification. Six skin samples with RNA integrity number (RIN) values above 6 were used for RNA-seq. mRNA selection, library construction, and sequencing were performed using the Illumina HiSeq X10 platform at NovelBio Company (Shanghai, China).

We used TRIzol reagent (Thermo, USA) to isolate total RNA from skin tissues according to the manufacturer's instructions. The RNA quality and quantity were tested using a NanoDrop spectrophotometer.

Primary Processing And Mapping Of Rna-seq Reads

Raw reads were filtered using NGS QC Toolkit v2.3.3 software, and we removed reads with an adaptor sequence, reads whose ends were of low quality (Phred quality value < 20) and reads with a length less than 25 bp after filtration. The sheep reference genome Oar_v4.0 was downloaded from the NCBI database (<http://www.ncbi.nlm.nih.gov/>). The clean data were mapped to the sheep reference genome with TopHat v2.0.11 software [26].

Assembly Of Transcripts And De Analysis

Gene expression was quantified using Cufflinks (v2.2.1) software [27] to obtain FPKM expression values. We performed read alignment and quantitated expression for each sample. The output files were sent to Cuffmerge with the reference annotation files. We used the Cuffdiff package of Cufflink (v2.2.1) software to analyze the DEGs between Tan sheep in the birth and Er-mao periods. Genes with fold change > 1 or < -1 and a P value < 0.05 were identified as DEGs.

PCA

The FPKM expression values for all of the annotated transcripts from transcriptions of the skin of six Tan sheep were used to perform PCA, which was implemented by ggplot2 in R (R v3.6.1, <https://www.r-project.org/>).

Functional Enrichment Analyses

KEGG analysis [28] was a database using for understanding functions and utilities of the biological system. Pathways enriched in genes with an adjusted P value < 0.05 were considered significantly enriched pathways.

Mrna Expression Analysis

One microgram of RNA from each Tan sheep skin sample was reverse transcribed using the PrimeScript RT with gDNA Eraser kit (Takara, Japan) according to the operating instructions.

The primers used for qPCR, which were designed with the NCBI database (<http://www.ncbi.nlm.nih.gov/>), are listed in **Table S1**. qPCR was conducted in an ABI 7500 system (Applied Biosystems, USA) with a reaction volume of 20 µL that included 2 µL of cDNA template, 10 µL of 2 × SYBR Green Master Mix (RR420A, Takara), and 0.5 µL each of upstream and downstream primers. The PCR amplification procedure consisted of a 10 s cycle of 95 °C, followed by 40 cycles of 95 °C for 15 s and 62 °C for 34 s. The fluorescence of the products after each cycle was detected. qPCR for each gene consisted of three

biological and technical replications, and the primer information is given in **Table S1**. ACTB was used as an internal reference gene. The $2^{-\Delta\Delta CT}$ [29] method was used to analyze relative mRNA expression.

Immunohistochemistry (the Sp Method)

Skin tissue sections were dehydrated and dewaxed with alcohol at different concentrations. After antigen repair, a 30 g/L H_2O_2 aqueous solution was added to block peroxidase for 10 min. The sections were incubated with normal goat serum albumin for 15 min, and each section was incubated with 50 μ L of secondary antibody at 37 °C for 4 h. After washing with PBS, 50 μ L of a biotin-labeled goat anti-rabbit IgG working solution was added to each section, and 50 μ L of a horseradish enzyme-labeled streptomycin working solution was added. After the addition of DAB, the sections were dehydrated, cleared and sealed.

Five slices were randomly selected, and 5 different fields in each slice were selected (400 $\times$). Image-Pro Plus 6.0 software was used to calculate the average absorbance indicating the expression factor from the positive signal strength and positive area in different visual fields from immunohistochemical staining of the skin tissue of Chinese Tan sheep in the birth and Er-mao periods. Twenty-five data points were counted for each period, and the results are expressed as the mean $\pm$ standard deviation. Statistical analysis was carried out with the R package, and one-way analysis of variance was used to analyze differences in the expression level of the same factor at the two different time periods.

Abbreviations

DEGs
Differentially expressed genes; KEGG:Kyoto Encyclopedia of Genes and Genomes; PCA:principal component analysis; DE:differential expression; H_2O_2 :Hydrogen peroxide; FPKM:Fragments per kilobase of transcript per million fragments mapped; qPCR:Real-time quantitative PCR; PBS:Phosphate Buffer solution; DAB:Diaminobenzidine.

Declarations

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Author Contributions

LYC performed most of the bioinformatics analysis. YCL, YHM, QM, DW, YHC, MZ, FL, DQH, JKW and LYC carried out the sample collection and RNA extraction. YCL participated in the immunohistochemical functional validation. LJ, YKL and JZT designed and coordinated the studies. YCL analyzed the data. LJ and YCL wrote the manuscript. All authors have read and approved the final manuscript.

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Availability of data and material

All the whole transcriptome data have been deposited in the Sequence Read Archive(<https://submit.ncbi.nlm.nih.gov/subs/sra/>; last accessed April 21, 2020) with the accession codes (BioProject ID: SUB7277087).

Ethics approval and consent to participate

We obtained written informed consent to use the animals in our study from the owner of the animals. Handling and sampling of the Chinese Tan sheep were carried out in full respect to animal welfare. All procedures including handling the Tan sheep samples were approved and implemented by the Laboratory Animal Care and Use Guide jointly developed by the Ministry of Agriculture of the People's Republic of China and the Institute of Animal Science of the Chinese Academy of Agricultural Sciences.

Consent for publication

Not applicable

Competing Interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Comparison of the Morphological observation of the skin tissue in two periods. (A) The wool on the birth period of the Chinese Tan sheep. (B) The wool on the Er-mao period of the Chinese Tan sheep. (C) The skin tissue on the birth period of the Chinese Tan sheep, 250μm. (D) The skin tissue on the Er-mao period of the Chinese Tan sheep, 250μm. (E) The comparison of the primary and secondary hair follicles diameters of the skin tissue on the birth period and the Er-mao period of the Chinese Tan sheep. (F) The

comparison of the primary and secondary hair follicles density of the skin tissue on the birth period and the Er-mao period of the Chinese Tan sheep PF: Primary hair follicle; SF: Secondary hair follicle; SG: Sebaceous glands. ** means a highly significant difference between groups($P<0.01$). * means significant difference between groups($P<0.05$).

Figure 2

Differential expression between the birth and Er-mao period. (A)PCA of the expressed genes of the skin tissue on the birth period and the er-mao period of the Chinese Tan sheep.BS means the skin tissue in the Birth period of the Chinese Tan sheep. ES means the skin tissue in the Er-mao period of the Chinese Tan sheep. (B) Volcanic plot of the genes on the birth and Er-mao period of the Chinese Tan sheep. (C) MAPK signaling pathway map. (D) The expression level of the differently expressed genes by qPCR. **means a highly significant difference between groups($P<0.01$). * means significant difference between groups($P<0.05$)

Figure 3

Immunohistochemical staining of the skin tissue on the birth period and the Er-mao period of the Chinese Tan sheep. (A) The immunohistochemical staining of the skin tissue on the birth period of the Chinese Tan sheep,20μm. (B) The immunohistochemical staining of the skin tissue on the Er-mao period of the Chinese Tan sheep,20μm.(C) The comparison of Fos expression of the skin tissue on the birth period and Er-mao period of the Chinese Tan sheep. SG: sebaceous glands; SF: secondary hair follicle; PF: primary hair follicle. **means a highly significant difference between groups($P<0.01$). * means significant difference between groups ($P<0.05$)

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