

Expression of fox-related genes in the skin follicles of Inner Mongolia cashmere goat

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Objective: This study investigated the expression of genes in cashmere goats at different periods of their fetal development.

Methods: Bioinformatics analysis was used to evaluate data obtained by transcriptome sequencing of fetus skin samples collected from Inner Mongolia cashmere goats on days 45, 55, and 65 of fetal age.

Results: We found that *FoxN1*, *FoxE1*, and *FoxI3* genes of the Fox gene family were probably involved in the growth and development of the follicle and the formation of hair, which is consistent with previous findings. Real-time quantitative polymerase chain reaction detecting system and Western blot analysis were employed to study the relative differentially expressed genes *FoxN1*, *FoxE1*, and *FoxI3* in the body skin of cashmere goat fetuses and adult individuals.

Conclusion: This study provided new fundamental information for further investigation of the genes related to follicle development and exploration of their roles in hair follicle initiation, growth, and development.

Keywords: Cashmere Goat; Real-time Quantitative Polymerase Chain Reaction Detecting System; Western Blot; *FoxN1*; *FoxE1*; *FoxI3*

INTRODUCTION

The sheep-raising industry has a long history in China, which is undoubtedly a country with developed sheep-raising regardless of quantity or types utilized. Inner Mongolia is one of the most important livestock breeding regions in China. This regions are also famous for its Cashmere goat, which is an indispensable part of the Inner Mongolia's animal husbandry. The precious animal fiber cashmere, produced in Inner Mongolia by Cashmere Goats, is rare and recognized for its outstanding quality in the whole world. The cashmere product obtained is slender, white-textured, and lustrous, which makes it a high-end textile material. Moreover, the textile products in which cashmere has been used as a raw material are not only light but also warm and smooth, which makes it popular and highly valued around the world. As a subsidiary structure of the skin, the hair follicle controls the growth of villi. Therefore, it has a direct impact on the yield and quality of cashmere. The different parts of the hair follicle are vary considerably in shape, but their basic structure is the same [1]. The follicle that governs the growth of the shag is called primary follicle. The growth of hair follicles is initiated by a signal from the dermal cells inducing the formation of hair buds from the epidermis. Then, hair buds release some factors that induce dermal fibroblasts to form dermal papillae, which then release a second signal to stimulate the proliferation and differentiation epithelial cells to form a complete hair follicle structure [2,3]. Transcription factors regulate gene expression and participate in the regulation of cell proliferation and differentiation.

Forkhead box (Fox) family of proteins is a class of DNA-binding domain transcription factors with a helix, first discovered in *Drosophila*. This protein family mediates a variety of important biological processes, such as DNA repair and embryonic development, and regulates the metabolic balance. So far, more than 20 Fox genes have been found in humans [4]. FoxN1 is a member of forkhead box protein family. It is mainly expressed in the thymic epithelial cells and skin keratinocytes, and plays an important role in different processes, such as the growth of thymus, T-cell proliferation, hair growth, and development of nails. There is evidence that the absence of FoxN1 in mouse leads to the development of nude mice, i.e. no thymus was formed, and they were hairless, with skin defects [5,6]. In the skin, FoxN1 is mainly expressed in the follicular epithelium and hair follicles [7]. The mutations of the *FoxE1* gene can lead to thyroid abnormalities, cleft palate, and inner nostril atresia, with thinning hair and a phenotype with two epiglottis [8]. The lack of FoxI3 prevents the downward growth of the hair follicles and obstructs the hair cycle [9]. The purpose of this examination was to determine the relative expression level of the differential genes FoxN1, FoxE1, and FoxI3 in the skin of cashmere goat fetuses and adult animals to provide new information for further investigation of the genes related to follicle development. In addition, we aimed to explore the roles of these genes in the initiation of hair follicle formation and their further growth and development at different periods of fetal development in cashmere goats.

MATERIAL AND METHODS

Material

Fetal samples from the body side skin of cashmere goat embryos were collected at the Aerbasi White Cashmere Goat Breeding Farm (Inner Mongolia, China) at 10 time points of fetal age (45, 55, 65, 75, 85, 95, 105, 115, 125, and 135 days). Samples were collected from 3 fetuses for each of these time points. For the collection of adulthood samples, three adult sheep were selected from the Inner Mongolia Cashmere Goat Breeding Farm, and skin samples were collected from the lateral body. Samples were collected every month on the same day, and samples were collected continuously for 12 months; Each time approximately 1 cm² of the skin collecting. All above-mentioned samples were cleaned with diethylpyrocarbonate water, numbered, quickly transferred into liquid nitrogen for freezing, and stored in a refrigerator at -80°C for future use. In this experiment, the breeding environment was in compliance with the standards relevant to an ordinary animal laboratory facility in China National Standard "Laboratory animal environment and facilities" (GB14925-2010). The feeding of and the experimental operations on animals were in accordance with the animal welfare requirements. The total RNA and proteins were extracted from each skin sample for

later use.

Methods

Tissue section of skin follicle of Inner Mongolia cashmere goats: The skin samples were washed with 1×phosphate buffer saline, dehydrated under different concentrations of alcohol and be transparent with Xylene, then put the skin into xylene: paraffin (1:1) overnight at 38°C. The paraffin was melted in 56°C about 4 to 5 h, then the skin samples were placed into melted paraffin wax. After wax solidification, it was cut into a trapezoid with knife, welded to the wood, slicing into pieces with 8 μm by microtome. The paraffin section was spread out and was placed on the glass slide. Then dry up in 37°C oven drying, the drying paraffin section were stained with HE and Saccpic methods and sealed with neutral resin. The photos were taken under a microscope [10].

Transcriptome analysis of differentially expressed genes in the skin of fetuses of cashmere goat at 45, 55, and 65 days of their fetal development: The transcriptome sequencing data of the skin from the studied fetuses of Inner Mongolia cashmere goat on days 45, 55, and 65 were used to screen the differentially expressed genes. They were analyzed online by conducting Venn analysis (<http://omics.pnl.gov/software/VennDiagram-Plotter.php>), and Gene Cluster and Tree View software were employed to perform the cluster analysis of differential genes.

Detection of the gene expression of FoxN1, FoxE1, and FoxI3 in skin hair follicles of Inner Mongolia cashmere goats: Real-time quantitative polymerase chain reaction (qPCR) detecting system was used to quantify the expression levels of the genes *FoxN1*, *FoxE1*, and *FoxI3* and mRNA in skin samples from cashmere goat collected at different fetal and adult stages. The expression status of the genes during hair follicle generation and development cycle was determined based on mRNA expression levels. First, using Primer premier 5.0 Software Design fluorescence quantitative primers, the primers of the house-keeping gene *β-actin* were designed by the Animal Genetics Laboratory at Agricultural University of Inner Mongolia according to the gene sequences *FoxN1*, *FoxE1*, and *FoxI3* registered

Table 1. Primers for real-time quantitative polymerase chain reaction detecting system analysis of the expression levels of cashmere goat *β-actin* and *FoxN1*, *FoxE1*, and *FoxI3*

Gene name	GenBank number	Sequence of primer	Products size (bp)
<i>β-actin</i>	NM_0010097	F: GGCAGGTCATCACCATCGG R: CGTGTTGGCGTAGAGGCTCTT	158
<i>FoxN1</i>	XM_0056932	F: GGTGAGAAGTCAAGGTCAA R: CCGCTGGTATGGATAGTG	176
<i>FoxE1</i>	XM_0040052	F: AGCTCATGGAAGCAACTCT R: ACCACATTTATGACCCAGCAG	120
<i>FoxI3</i>	XM_0056867	F: GAAAGCCAGATGGAGACAA R: TGCTACTACCGACGCTGA	171

in GeneBank. Then, the primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The primer sequences are listed in Table 1.

The Primer Script™ RT reagent Kit (TaKaRa Code: RR 047A) (TaKaRa, Kyoto, Japan) was used in the analyses. Different reverse transcription reagents were added depending on the concentration measured, and the total RNA was reversely transcribed. Real-time qPCR was performed using the real-time fluorescence quantitative instrument Agilent (USA). The expression levels of *FoxN1*, *FoxE1*, and *FoxI3* genes in samples of skin tissues collected at different time points of the fetal and adulthood periods of cashmere goats. A volume of 20 µL of the reaction system contained 10 µL of 2X SuperReal PreMix, 1 µL of diluted reverse transcription product, 2 µL of each specific primer (10 µmol/L), 0.4 µL of ROX (50x), and 6.6 µL of ddH₂O. The PCR conditions were as follows: a pre-denaturation for 3 min at 95°C, followed by 40 cycles of 30 s denaturation at 94°C, 30 s at the optimum annealing temperature, 60 s of extension at 95°C, and 0.5 min final extension at 55°C. The threshold cycle (Ct value) was automatically calculated from PCR amplification plots in which fluorescence was plotted against the number of cycles; each sample was analyzed in three replicates.

The $2^{-\Delta\Delta Ct}$ method was applied to conduct a relative quantitative analysis of the real-time PCR data. The fetal skin samples collected on day 45 were used as calibration samples. The following formulas (1 to 3) were used for the calculations:

$$\begin{aligned} \Delta Ct \text{ (calibration sample)} \\ = Ct \text{ (calibration sample target gene)} \\ - \text{calibration sample } \beta\text{-actin} \end{aligned} \quad (1)$$

$$\begin{aligned} \Delta\Delta Ct \\ = (Ct \text{ of the sample of the target gene} \\ - Ct \text{ of the housekeeping gene}) - \Delta Ct \end{aligned} \quad (2)$$

$$\text{The relative level of gene expression} = 2^{-\Delta\Delta Ct} \quad (3)$$

All tests were repeated three times. The experimental data obtained were expressed as mean±standard deviation. Finally, the data were used to conduct variance analysis using SAS9.0 software with a level significance at $p<0.05$. Similarly, the $2^{-\Delta\Delta Ct}$ method was also applied to calculate the PCR data of the adulthood samples. The samples from the skin of adult white cashmere goat of Inner Mongolia gathered in March were used as calibration samples. The following formula were used for the calculations (4 to 6):

$$\begin{aligned} \Delta Ct \text{ (calibration sample)} \\ = Ct \text{ (calibration sample target gene)} \\ - Ct \text{ (housekeeping gene)} \end{aligned} \quad (4)$$

$$\begin{aligned} \Delta\Delta Ct \\ = (\text{the sample of the target gene Ct} \\ - \text{the sample of } \beta\text{-actin Ct}) - \Delta Ct \end{aligned} \quad (5)$$

$$\text{Expressed relative gene amount} = 2^{-\Delta\Delta Ct} \quad (6)$$

All tests were repeated three times. The experimental data obtained were expressed as mean±standard deviation. Finally, variance analysis of the data was conducted using SAS 9.0 software with a level significance at $p<0.05$.

Detection of the expression of FoxN1 and FoxE1 proteins in the skin hair follicles: The genetic information in the DNA sequence of an organism is transcribed and translated, and it is ultimately realized in the form of synthesized biologically active protein molecules. Because genes *FoxN1*, *FoxE1*, and *FoxI3* are not goat-derived antibodies, we chose a highly homologous primary antibody derived from rabbit or murine. However, the primary antibodies against FoxI3 originating from rabbit or murine are quite different from the amino acid sequence of sheep FoxI3, which makes them unsuitable for the Western blot analysis. Therefore, in this experiment, Western blot was used only to detect FoxN1 and FoxE1 in the skin samples from the body side of cashmere goat embryos collected at 10 time points of fetal development (45, 55, 65, 75, 85, 95, 105, 115, 125, and 135 days). Samples from three fetuses were analyzed for each time point. In addition, we established the expression levels of the aforementioned proteins in skin tissue samples from adult sheep collected at four representative time points: June, September, December, and March. Our findings provide some clues and the basis for further exploration of the functions and mechanisms of action of genes *FoxN1* and *FoxE1* in hair follicle development and growth process at different developmental time points.

RESULTS

Tissue section of skin follicle of Inner Mongolia cashmere goats

Early in the developmental process of the formation of the structure of cashmere goat hair follicle, we conducted a morphogenesis study and found that on gestational day 45 fetal epidermis was the only layer of flat cells that did not form primary follicles [11]; Inner Mongolia Cashmere Goats develop the complete structure of their epidermis from gestational day 45 to gestational day 55, the primary follicles have not yet begun to develop during this period. Keratinocytes were aligned neatly on epidermal base (Figure 1A). During the development of the fetus from day 55 to day 65, the hair buds increase in length significantly, penetrating into the cells of the dermis. The secondary follicle primordium can be observed from the fetal side of the body (Figure 1B); During the development of the fetus from day 65 to day 75, the secondary follicle primordium

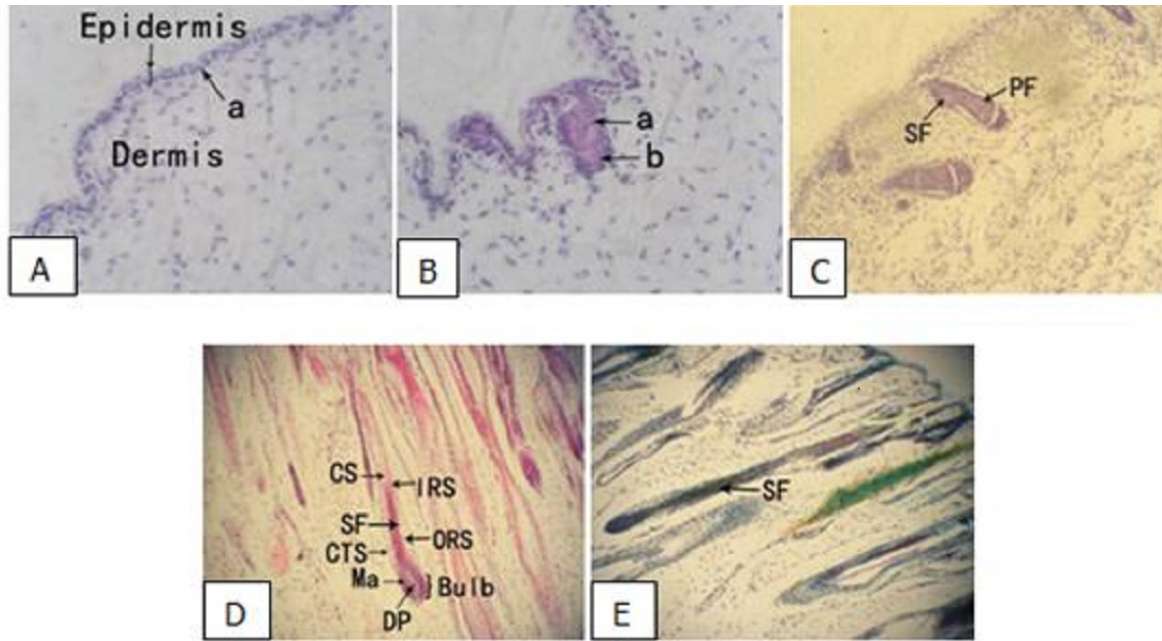


Figure 1. Changeable process hair follicle morphogenesis of the Inner Mongolian cashmere goat in the fetal period [10]. (A) Fetal period of 55 day (400×); a, hair buds precursor. (B) Fetal period of 65 day (400×); a, growing hair bud; b, cap like structure of dermal fibroblasts. (C) Fetal period of 75 day (200×); SF, secondary follicle; PF, primary follicle. (D) Fetal period of 125 day (100×); DP, dermal papilla; Ma, hair matrix; CTS, connective tissue sheath; ORS, outer root sheath; IRS, inner root sheath; CS, cashmere shaft. (E) Fetal period of 135 day (100×).

can be observed from the fetal side of the body, secondary follicles begin to occur (Figure 1C). Most secondary follicles develop their complete structure by the 125th day of fetal growth, and hair matrix cells surround the dermal papillae. Both dermal papillae and primary hair follicles have an oval shape which facilitates the penetration of villi through the body surface (Figure 1D). On day 135, a part of the hair follicles are mature, and the villi have pierced through the body surface [10] (Figure 1E). At birth, all primary follicles are mature,

whereas only a small number of secondary follicles are mature at that stage. Within 18 months after birth, increasingly more secondary follicles mature, and at the age of six months, hair fibers have emerged from all secondary follicles, meaning that only the secondary follicles finish their development six months after birth. The growth pattern of cashmere wool showed a strong seasonal variation of adult. In a cycle, hair follicle activity includes anagen, catagen, and telogen [12] (Figure 2).

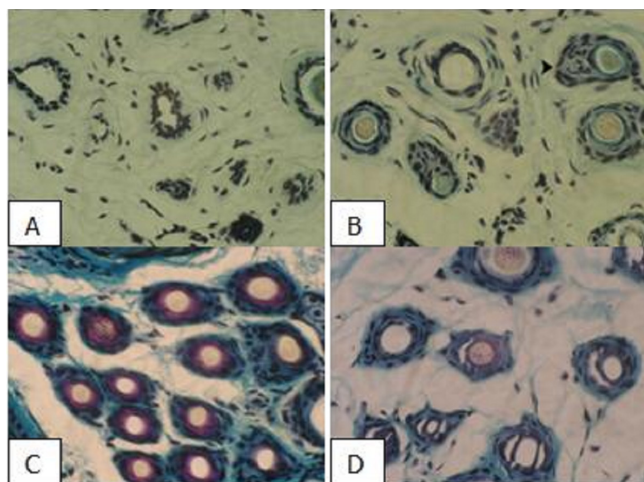


Figure 2. Cycling of follicle on Inner Mongolia cashmere goat [11]. (A) Telogen (January). (B) Early anagen (April). The arrow indicate the finger cell group in active. (C) Anagen (August). (D) telogen (November). Original magnifications, 400×.

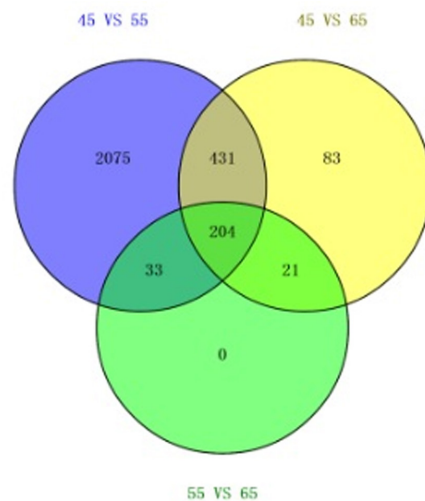


Figure 3. Venn diagram analysis of differentially expressed genes on days 45, 55, and 65 of the fetal period.

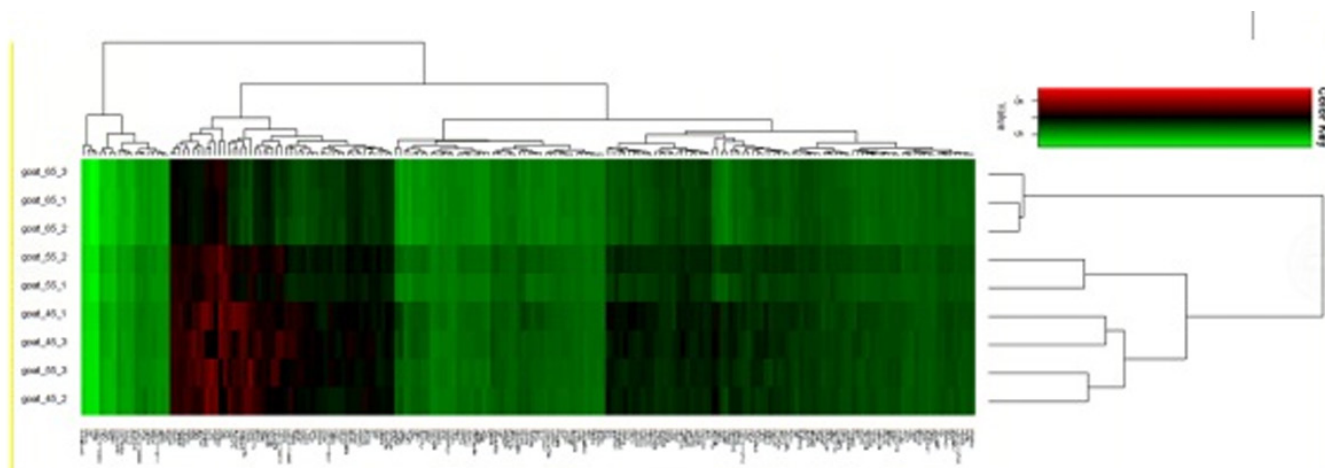


Figure 4. Cluster diagram of 2,081 differentially expressed genes on days 45, 55, and 65 of the fetal period. In the graph, the green color indicates high gene expression level, and the red color indicates low gene expression level, each column represents a gene, each row is compared with a sample of different periods, the left side from top to bottom are goat-65-3, goat-65-1, goat-65-2, goat-55-2, goat-55-1, goat-45-1, goat-45-3, goat-55-3, goat-45-2, the increase in gene expression is expressed in color from green to red.

Screening of fetal skin tissues for differentially expressed genes on days 45, 55, and 65

Comparison was conducted between the gestational samples collected on days 55 and day 45, day 65 and day 45, and day 55 and day 65. We established the presence of up to 2,847 differentially expressed genes ($p \leq 0.05$). The overlap analysis of these differences in the gene expression of the three parts (45 vs 55, 45 vs 65, 55 vs 65) resulted in the generation of a total of 204 expressed genes (Figure 3). The results of the cluster analysis performed in nine of the samples show that most of the samples cluster together according to the development period of hair follicles with only a few exceptions (Figure 4).

Basing on the evidence of some recent reports of studies on hair follicle development at home and abroad, we hypothesized that these three genes of the Fox gene family (*FoxI3*, *FoxN1*, and *FoxE1*) are probably involved in the growth and development of the follicle and the formation of the hair. Of them, *FoxI3* and *FoxN1* were included in the 204 differentially expressed genes that were common in all three periods. Therefore, *FoxI3*, *FoxN1*, and *FoxE1* were selected as candidate genes for conducting research on their expression level in skin follicles.

Expression of genes *FoxN1*, *FoxE1*, and *FoxI3* in skin tissue of Inner Mongolia cashmere goat

Relative expression level of FoxN1, FoxE1, and FoxI3 genes in cashmere goat fetal skin hair follicles: The genes *FoxN1*, *FoxE1*, and *FoxI3* were expressed in the skin tissue of cashmere goat fetus in all 10 experimental time points (Figure 5). The expression level of gene *FoxN1* did not change significantly ($p > 0.05$) from day 45 to day 95, but there was a significant increase in its expression level from the beginning of day 95 ($p < 0.05$). The peak expression level occurred on day 135, whereas the expression level on day 115 was slightly lower, but both of them

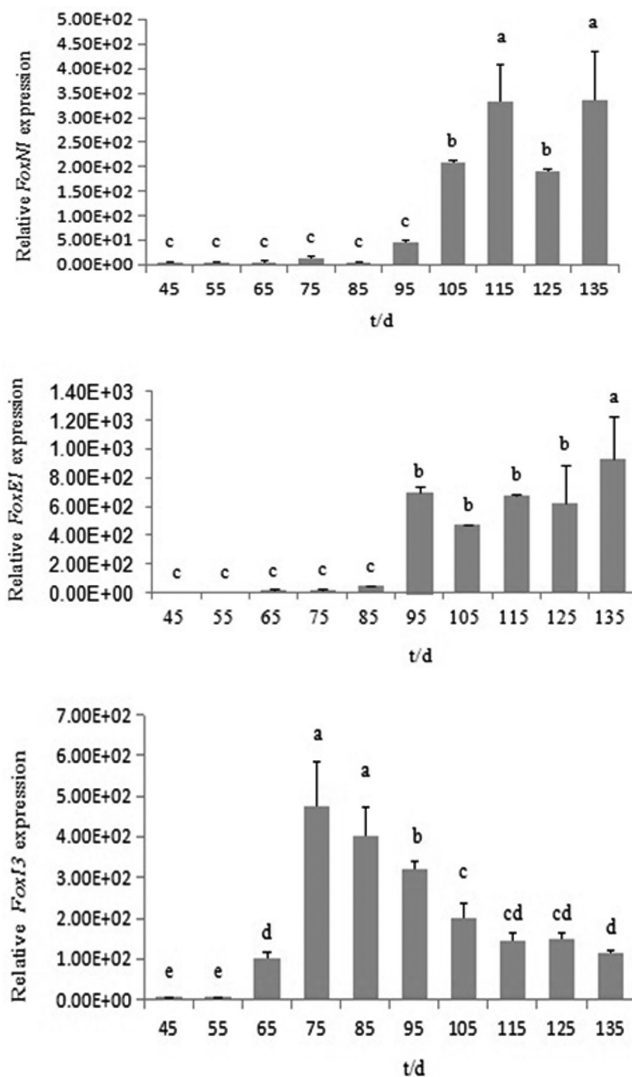


Figure 5. Relative expression levels of *FoxN1*, *FoxE1*, and *FoxI3* genes during the fetal period. The different letters indicate significant difference at $p < 0.05$.

were significantly higher than the expression levels of the other fetal periods ($p < 0.05$). The level of expression of *FoxE1* gene showed an overall upward trend, although there was no significant change in the expression level from day 45 to day 85 ($p > 0.05$). The expression level on day 95 was relatively higher than those at other time points of fetal age, but it reached its peak value on day 135, which was significantly higher than those of the other fetal development time points ($p < 0.05$). The trend for *FoxI3* gene expression in fetal skin tissue of cashmere goat in the 10 time points, with a peak on day 75, was opposite to the trend observed for *FoxN1* and *FoxE1*. The gene expression level on day 85 was lower than that on day 75 but was still significantly higher than that of days 45, 55, and 65 ($p < 0.05$). The expression level of gene *FoxI3* in the hair follicles of the skin on day 65 was significantly higher than that on day 45 ($p < 0.05$).

Relative expression levels of FoxN1, FoxE1, and FoxI3 genes in skin hair follicles of adult cashmere goats: FoxN1, FoxE1, and

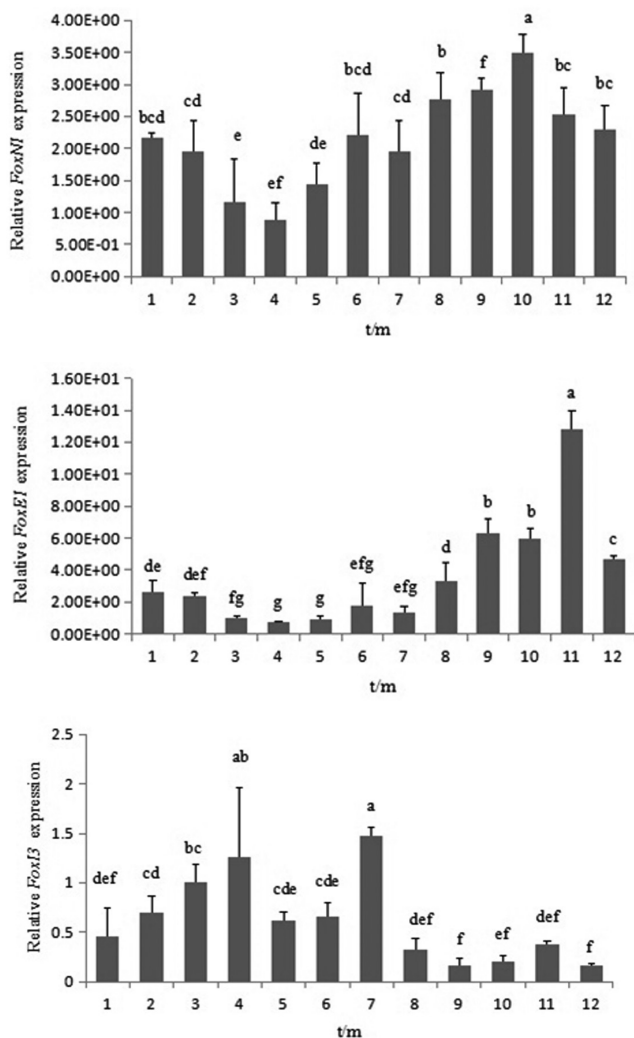


Figure 6. Relative expression levels of *FoxN1*, *FoxE1*, and *FoxI3* genes during the 12-month period. The different letters represent significant difference at $p < 0.05$.

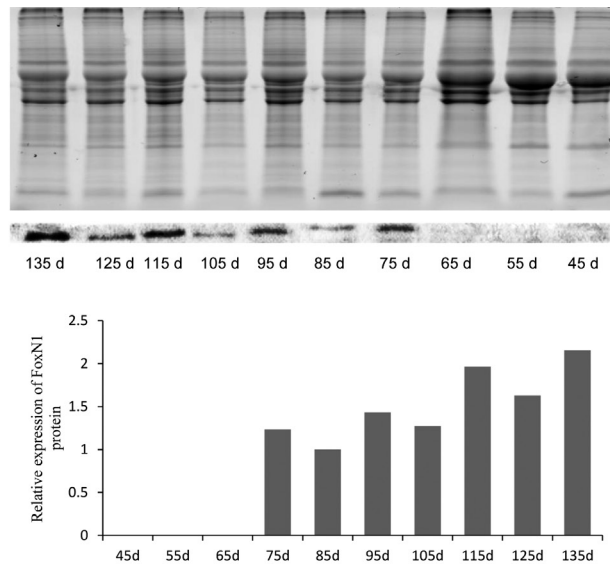


Figure 7. Expression of FoxN1 protein in the fetal period.

FoxI3 genes were expressed in the skin of adult cashmere goats at different stages of their age (Figure 6). The expression level of *FoxN1* gene was significantly higher than those of other stages ($p < 0.05$). The trend of gene *FoxE1* expression level was basically consistent with that of the expression level of *FoxN1* gene. The expression level of *FoxI3* gene reached its highest value in July and was significantly higher than the expression levels in March, September, and December ($p < 0.05$).

Expression of FoxN1 and FoxE1 proteins in the skin hair follicles of Inner Mongolia cashmere goats

Expression of FoxN1 and FoxE1 proteins of fetus skin hair follicles

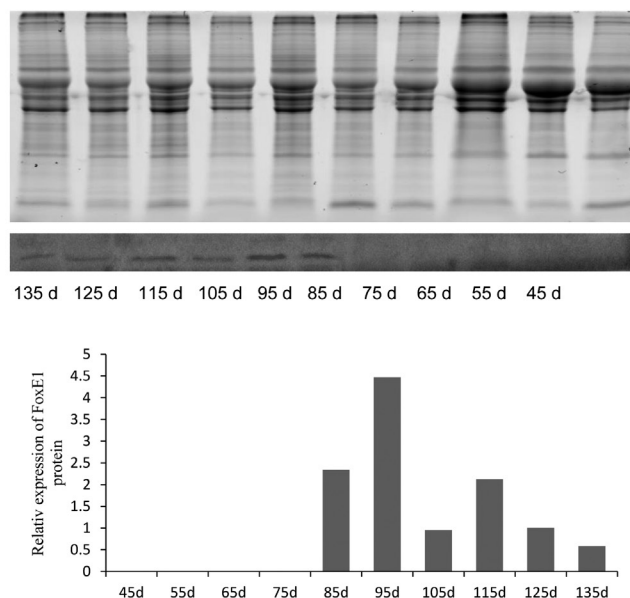


Figure 8. Expression of FoxE1 protein in the fetal period.

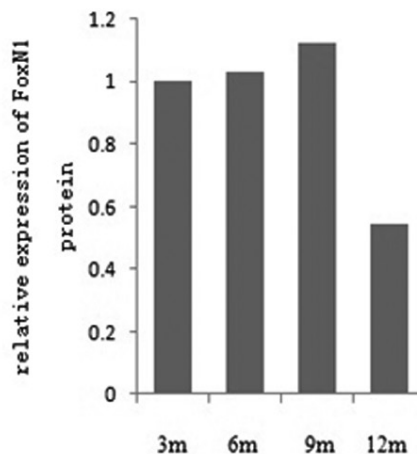
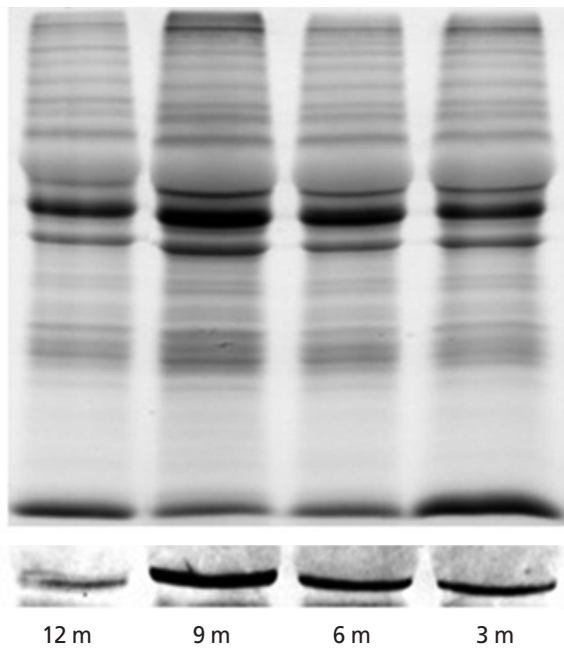


Figure 9. Expression of FoxN1 protein in the different growth periods.

of goats: Western Blot was used to detect the protein expression of FoxN1 and FoxE1 in the skin hair follicles. The results showed that these two proteins were expressed in the hair follicles of the fetus skin in different stages of the development of the Inner Mongolia cashmere goat. The analysis results represented the relative amount of the protein of interest in each of the samples. FoxN1 protein expression can detect from gestational day 75, and the expression level had an overall upward trend, in which gestational day 135 had an expression level that was relatively higher than those on the other gestational days (Figure 7). Protein FoxE1 expression was be detected from

gestation day 85, reaching the maximal relative expression level on gestational day 95 (Figure 8).

FoxN1 and FoxE1 protein expression in the skin hair follicles of adult Inner Mongolia cashmere goats: Western blot was used to detect the expression of FoxN1 and FoxE1 proteins in skin tissue from adult sheep gathered in March, June, September, and December. The results indicated that FoxN1 and FoxE1 proteins were differentially expressed in the skin tissue of adult Inner Mongolia Cashmere goats at different adulthood periods. The results represented the relative amount of the protein of interest in the samples. We found that the relative

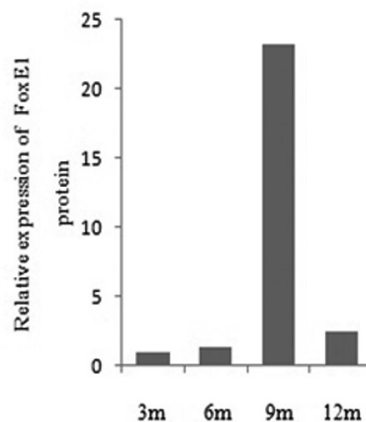
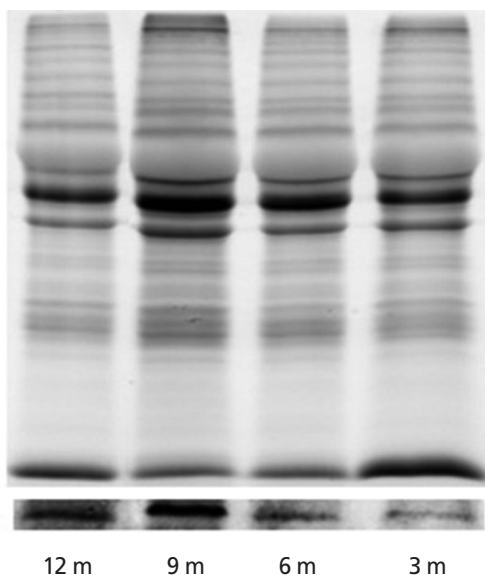


Figure 10. Expression of FoxE1 protein between different growth periods.

expression level of protein FoxN1 in September is higher than those of protein FoxN1 in March, June, and December (Figure 9). On the other hand, the relative expression level of protein FoxE1 in September was far more elevated than the expression levels of protein FoxN1 in March, June, and December (Figure 10).

DISCUSSION

Screening of differentially expressed genes in fetal skin tissues of cashmere goats on day 45, 55 and 65

Venn diagram analysis was conducted on the continuously up-regulated genes in the fetal skin samples of cashmere goats on days 45, 55, and 65 (Figure 3). The comparative gene annotations on day 55 vs day 45, day 65 vs day 45, and day 65 vs day 55, revealed the presence of a total of 204 genes. This paper focuses on *FoxN1* and *FoxI3* was contained in these 204 genes. The cluster analysis of the differentially expressed genes in the different periods (Figure 4) showed that biological repeatability is the best in 65 days. On 45 and 55 days clusters of two samples were formed, which means the gathered samples were reliable. As can be seen from Figure 2, some genes showed a trend of up-regulation at these three time points, and *FoxN1* and *FoxI3* were two of them. In an earlier study *FoxN1* was mainly expressed in the follicular epithelial cells and the skin hair follicle [8]. Other studies showed that patients suffering *FoxE1* mutations had defective hair follicles, which suggests that *FoxE1* may be involved in hair follicle morphogenesis. Therefore, the candidate genes *FoxN1*, *FoxI3*, and *FoxE1* in the hair follicles of cashmere goats were selected for the experiments in our research.

Relationship between *FoxN1*, *FoxE1*, and *FoxI3* genes and the origin and development of the hair follicle

According to previous research reports that Inner Mongolia cashmere goats develop the complete structure of their epidermis from gestational day 45 to gestational day 55. Nevertheless, the primary follicles have not yet begun to develop during this period. At the age of 55 days to 65 days, the formation of primary follicle. The formation of secondary follicles begins from gestational day 65 to day 75. Then, the primary follicles on the body side grow fast from gestational day 95 to gestational day 115. In addition, the secondary follicles on the body side also grow fast from gestational day 105 to gestational day 125. During the process of changes of follicle cycling, the upper section of hair follicles, i.e., the sebaceous glands and the part above the bulge remain stable, and apoptosis and regeneration do not occur. The lower section of the follicles, namely the hair follicles and the hair bulb part that is below the bulge, experiences morphological changes, such as growth, anaplasia, and diapause. Nonetheless, the changing patterns of the primary and secondary follicles in cashmere goat are quite different and

dependent on the seasonal changes. No considerable changes are observed in the traits of the primary follicles as most of them remain active, and only a part enter the catagen phase. However, the secondary follicles show strong regularity that is based on the seasonal light cycle. From April to November, the secondary follicles gradually enter a period of growth and then of catagen (from December to January), before entering the telogen phase (from February to March). The stem cells are activated during the growth period, and the cells near the dermal papilla begin proliferating, forming new hair bulbs. After the formation of hair balls, the inner root sheath and the hair shaft begin to differentiate. It is in this period that the hair follicles begin to grow, and hair balls proliferate rapidly, and the duration of the growth period determines the length of the hair shaft. However, the inner root sheath then degenerates at the funnel part. Importantly, extensive apoptosis occurs in the catagen phase, and the differentiation is stopped. During this period, the hair bulbs become wider and larger, the hair roots go up, the claviform hair is formed, and the dermal papillae get in contact with the epithelia. In the telogen phase, the clavellate hair is anchored, apoptosis is terminated, the dermal papillae are connected with the bulge stem cell niches, and the hair follicles become dormant and enter the telogen stage. In this period, there is no significant proliferation, apoptosis, and differentiation, and the stem cells are located near the dermal papillae [13,14]. These processes are regulated by a series of signaling molecules, including bone morphogenetic protein (BMP) family, homeobox genes, fibroblast growth factor-5 (FGF5), that are needed to elicit the starting signal, the signal to maintain the growth of hair follicles, and the one initiating inhibition of the hair follicle growth. According to the evidence presented in reports published recently, the major upstream regulatory signaling pathways of *FoxN1* are the Wnt pathway and the BMP pathway. It is speculated that the multifunctional developmental regulator BMP-4 may be used as a signaling molecule to induce the expression of *FoxN1*. Earlier studies in mice revealed that both the first layer of skin basal cells and some of the epidermal basal cells express *FoxN1*, whereas the starting signal for initiation of the development of hair follicles comes from the bottom-cell base [15], and the cause of the nude phenotype is a gene mutation of *FoxN1*, which leads to its loss of functionality [16]. *FoxN1* mutations in humans cause immune deficiency, alopecia, nail dystrophia etc. Our results showed that the gene expression level of *FoxN1* increased significantly on gestation day 95 ($p < 0.05$). Previous research on the morphogenesis of secondary hair follicles of cashmere goats evidenced that the inner root sheath of the primary follicle, the outer root sheath, and the hair shaft developed until gestational day 95. At this time point of our study, a small amount of inner and outer root sheath could be observed from the side of secondary follicles, and the villi also began to form. On day 135, the development of the primary

follicle was substantially complete, and part of the secondary hair follicles matured and the villi pierced the body surface, which indicated that *FoxN1* may be involved in the differentiation of the inner root sheath, cortex, and medulla. The cyclical growth results showed that the expression of *FoxN1* gene was overall elevated from April to October and reached a peak value in October that was significantly higher than those established at other stages ($p < 0.05$). Further, from November to March, it declined continuously.

The expression trend of *FoxN1* gene and the development cycle of hair follicles were basically consistent. Previous research on more mature follicles and growing hair follicles revealed that *FoxN1* transcription occurred predominantly in the mature areas of the hair follicles, hair shaft, inner root sheath, and the outer root sheath [17]. Therefore, it could be speculated that *FoxN1* plays an important role in the hair follicle growth. Reportedly, the mutations of *FoxE1* gene caused Bamforth-Lazarus syndrome. The patients' epiglottis was hypoplastic and bifid with sparse and spiky hairs, but the larynx was of normal diameter; the jaw was retrognathic [18]. Later, Clifton-Bligh et al [19] found that this was due to a mutation of codon 65, a substitution of alanine for valine. We established the expression level of *FoxE1* from gestational day 95 to day 135 was relatively higher than those of the other periods. Moreover, its expression level showed an overall upward trend with the increase of gestational age, suggesting that *FoxE1* gene is involved in hair follicle morphogenesis and plays a role in the development and maturation of hair follicles. It was expressed throughout the periodical growth process of the hair follicle, with a peak relative expression level in November. These findings are consistent with the reported conclusion that *FoxE1* plays an important role in the embryonic development, cell growth, and differentiation. Therefore, it can be speculated that *FoxE1* may be involved in the regulation process of periodical growth of the hair follicle.

Using the chip technology, researchers compared the DNA fragment of hairy and hairless dogs. They discovered that a mutation in the hairless dogs had been inherited from a unique ancestral, thus giving the now well-known name of the gene *FoxI3*. In dogs, *FoxI3* haploinsufficiency leads to poor embryo development, which is characterized by an almost entire absence of hairs. In a mouse model, *FoxI3* was found to show highly dynamic expression patterns in hair formation and cycle. In addition, the lack of *FoxI3* prevents the downward growth of the hair follicles and hinders the progress of the hair cycle. Therefore, we can be certain *FoxI3* regulates many aspects of hair follicle development and dynamic equilibrium. Vera Shirokova located the *FoxI3* gene downstream of ectodysplasin (EDA) signaling pathways. And Noggin, BMP and EDA signaling molecules play an important role in the early developmental stages of follicle board. Thus, we further speculate *FoxI3* gene may influence the origin and development

of hair villi through interaction with other signaling molecules. Nevertheless, its role in the regulation of generation and development of the hair follicle needs further investigation. Our experiments demonstrated that the expression level of *FoxI3* gene peaked on day 75, the level on day 65 was significantly higher than the expression level on day 45 ($p < 0.05$). According to our results, the primary follicles in the Inner Mongolia cashmere goats begin to form from gestational day 55 to day 65, the secondary follicle growth occurs from day 65 to day 75, which indicates that *FoxI3* gene may play a role in the secondary follicles initiation. *FoxI3* was expressed in the skin tissue of adult cashmere goats all the year round, and its maximum relative expression level occurs in July. The results of our study show that the boundaries of each period are not absolute. The growth of the secondary follicles of Liaoning and Inner Mongolia cashmere goats was most active from July to November, with a peak in September. In November, the follicle cells began to die and catagen features appeared, and the processes in this month may be considered the transition from the growth phase to the catagen stage of secondary follicles. In January, follicles transit from catagen to their period of minimum activity – telogen, but in April, they transit back from this resting phase (telogen) to their growth phase (anagen). We hypothesize that *FoxI3* may promote the growth of hair follicles in the anagen.

Relationship between *FoxN1* and *FoxE1* proteins and the cyclical growth of the hair follicles

The production of goat cashmere and the quality of cashmere fibers are associated with the level of keratin. To study the important economic traits of cashmere sheep, the scholars at home and abroad mainly focus their investigations on the keratin gene family. Keratin is the most abundant protein in hair follicles and plays an important role in maintaining their structure. *FoxN1* is a conservative transcription factor that regulates the expression of keratin in the skin. The absence or mutation of *FoxN1* can cause hair shaft curl in the hair follicles due to cornification defects, which leads to the loss of its ability to penetrate the epidermis and form normal hair. The loss of the proteolytic activity of *FoxN1* in humans and mice results in hypotrichosis, immunodeficiency (absence of thymus and T cells) and skin defects [20,21]. *FoxN1* is expressed in the initial stage after the mitosis of hair follicle stromal cells. In the meantime, the stromal cells tend to differentiate in the direction of internal root sheath.

Based on the results of real-time qPCR of our examination reported here, we can conclude that the relative expression of the gene *FoxN1* from gestation day 45 to gestation day 65 is relatively low since no expression of the respective protein was detected. The protein expression level exhibits an upward trend on gestation day 75. After day 75, the hair follicles grow and mature, and the structure of the hair shaft is gradually formed.

Presumably, the protein FoxN1 can maintain the normal hair structure by regulating the expression of hair keratin. In the cyclical growth phase, the expression level of protein FoxN1 in September was higher than its levels in the other three periods. While the hair follicles entered the active period in September. In this period, the hair follicles of adult goats appear, develop, and rapidly produce a large amount of cashmere. Skin to express a large number of keratin and keratin associated protein. It is speculated that FoxN1 may play a catalytic role in the synthesis of keratin.

In previous studies, the expression of FoxE1 was found to be periodically suppressed in the quiescent stage, and then it induced the initiation of a new growth period. The hair defects that can be observed are due to the abnormality in the hair follicle morphology, leading to the loss of the potential of hair follicles to penetrate correctly through the dermis and the subcutaneous tissue, which causes a deviation of the direction. Studies have found that protein FoxE1 is under expressed in the outer root sheath around the hair bulb and is a downstream target signal of Shh, participating in the later hair follicle morphogenesis. The lack of FoxE1 leads to abnormal extension of hair follicles to the depth of the dermis. The experimental results reported in this paper showed that the relative expression level of protein FoxE1 on gestation day 95 was higher than those in the other time points of gestational age. On gestation day 95, the stromal cells located at the end of the hair follicles surrounded the dermal papilla cells forming the hair bulbs. Meanwhile, the inner root sheaths and the outer root sheaths of some hair follicles could be observed. We speculate that the protein FoxE1 regulates the development of late hair follicle morphogenesis by interacting with a variety of signaling pathways. The experimental results reported in this paper showed that protein FoxE1 is expressed in March, June, September, and December. Presumably, FoxE1 may be involved in the cyclical growth of hair follicles. Its expression level in September is higher than the ones in the other months, because September is a relatively productive period in terms of hair follicle growth. Importantly, FoxE1 may be involved in the process of formation of the hair follicle.

CONCLUSION

This study is based on the previously acquired transcriptome data of Inner Mongolia Cashmere Goat fetal skin samples on gestational days 45, 55, and 65. Using bioinformatics analysis, we identified 204 differential co-expressed genes during the three time points, including *FoxN1* and *FoxI3* and another Fox gene family member *FoxE1*. By using *FoxN1*, *FoxI3*, and *FoxE1* as candidate genes, we carried out studies on the expression of these genes in the hair follicles of the skin. Further, using real-time qPCR and Western blot, we analyzed the expression levels of mRNA and protein during the period of the devel-

opment and cyclical growth of hair follicles in the skin of cashmere goat. Real-time qPCR results show that there is a general trend of an increase in the expression levels of *FoxN1* and *FoxE1* in the fetal period, and their maximum expression level is reached on gestational day 135. On the other hand, the expression level of gene *FoxI3* is gradually elevated starting from gestation day 45 and achieving its maximum on gestational day 75; from gestation day 85 onwards, the expression level slowly declines. The trends of changes in the expression of *FoxE1* and *FoxN1* are in line with the cyclical changes in the growth cycle of the hair follicles. The expression of gene *FoxI3* reaches its maximum in July, followed by that in April; the expression levels in both months are significantly higher than those in other periods ($p < 0.05$). We speculate that genes *FoxN1*, *FoxI3*, and *FoxE1* may have a regulatory effect on the growth and development of hair follicles and their cyclical growth process. The Western blot results show that the expression level of protein FoxN1 in general increases from gestation day 75 to gestation day 135, reaching its maximum on the later. The expression level of protein FoxE1 is the highest at the gestational age of 95 days, and in the period after 105 days of gestation, its expression begins to decrease. In the growth cycle, protein FoxN1 and protein FoxE1 are expressed in the hair follicles in the stages anagen, telogen, and catagen. The expression levels of the two proteins reach their peaks in September, during the active period of hair follicle growth. Basing on these findings and the real-time qPCR results, we speculate that proteins FoxN1 and FoxE1 may be involved in the late stage of unidirectional growth downwards of hair follicles and hair shaft formation.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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