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Comparative study on seasonal hair follicle cycling by analysis of the transcriptomes from cashmere and milk goats

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ABSTRACT

Guard hair and cashmere undercoat are developed from primary and secondary hair follicle, respectively. Little is known about the gene expression differences between primary and secondary hair follicle cycling. In this study, we obtained RNA-seq data from cashmere and milk goats grown at four different seasons. We studied the differentially expressed genes (DEGs) during the yearly hair follicle cycling, and between cashmere and milk goats. WNT, NOTCH, MAPK, BMP, TGFβ and Hedgehog signaling pathways were involved in hair follicle cycling in both cashmere and milk goat. However, Milk goat DEGs between different months were significantly more than cashmere goat DEGs, with the largest difference being identified in December. Some expression dynamics were confirmed by quantitative PCR and western blot, and immunohistochemistry. This study offers new information sources related to hair follicle cycling in milk and cashmere goats, which could be applicable to improve the wool production and quality.

1. Introduction

Since several centuries ago, China has a long history of goat (*Capra hircus*) breeding, and it owns abundant goat breeding resources such as cashmere goat and milk goat. The goat industry is a vital constituent of Chinese animal husbandry, with previous researches showing that China is the largest producer of cashmere fiber around the world [1]. Cashmere goat is the most famous livestock around the world but is only found in specific areas in Asia, including northern China, Mongolia, Afghanistan and Iran. However, milk goat is widely distributed in Asia, Africa, Europe and America [2]. Previous research shows that the fleece of Cashmere goats is made up of two distinct fibers: guard hair and cashmere undercoat, but in milk goat is mainly made up of guard hair [3]. Different hair is produced from the hair follicle; guard hair produced by primary hair follicle, and the undercoat which is the source of cashmere products such as pashmina or sweater is produced by secondary follicles [3–5]. Although the composition of guard hair and cashmere undercoat is very similar, but the structure, development pattern and some other characters in secondary hair follicle are significantly different from the primary hair follicle. Moreover, various researches have indicated that the post-natal hair follicle of mammalian species undergoes a cycling of anagen (active), catagen (quiescent) and telogen (inactive) [6–8]. The guard hair in cashmere and milk goat grows faster in summer and autumn and sheds in spring. However, the development cycle of undercoat hair follicle and the fiber shedding pattern in Cashmere goat are significantly different from guard hair [3–5,9–11].

To date, most studies related to goat-hair follicle development have focused on two aspects, namely, morphology and molecular genetics [3,12–15]. At first, in the area of morphology, the follicle densities, secondary to primary follicle (S/P) ratios and follicle number indices (FNI) of fiber-bearing primary (Pf) and secondary (Sf) follicles have

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been shown to be different in primary and secondary hair follicle development. For example, Parry and his colleagues have found that during the skin follicle development in Australian cashmere goat, all primary follicles were present but only few secondary follicles were mature at birth, and secondary follicles increased significantly faster than primary follicles [15]. Rischkowsky and Momen [3] have compared hair follicle density and activity between male and female cashmere goats, the results revealed that S/P ratio, follicle density and activity in male are significantly higher than female [3]. Moreover, in another research, we found that the number of primary and secondary follicles were significantly different between cashmere and milk goat. In cashmere goat, there are 21 secondary hair follicles and only 2 primary hair follicles per square millimeter. However, in each square millimeter milk of goat skin samples, there are > 30 primary hair follicles (unpublished data). These studies indicate that the primary and secondary hair follicle development is different in morphology.

Second, in molecular genetics, there are several studies about hormonal regulation in hair follicle development [4,12,14,16,17]. Ibraheem and his colleagues (1994) did some in vitro experiments about secondary hair follicle growth response to prolactin and melatonin in cashmere goat, and found that prolactin and melatonin act directly on secondary hair follicle of the cashmere goat to stimulate the elongation of the hair shaft. This result suggests the seasonal control of the hair growth cycle and entails the involvement of prolactin and melatonin [14]. In addition, there are also a large number of studies on genes which play important roles in Cashmere goat secondary follicle development [12,13,18]. Zhang (2014) has compared the *Hoxc9* gene in different goat species, and the results revealed the gene to be significantly differentially expressed in different species. Moreover, Geng (2014) did a lot of studies on *Lhx2* gene and found that the highest expression was observed at the anagen stage, while the lowest expression was detected at the telogen stage [12]. These findings provided a better understanding of the function of *Lhx2*, and suggested that the cyclic expression of *Lhx2* might play important roles during secondary hair follicle development in Cashmere goat.

With the development of sequencing and bioinformatics technologies, high throughput RNA sequencing (RNA-seq) provides a platform for measuring large-scale gene expression pattern [19–21]. In order to identify DEGs and novel transcript units, RNA-seq has been recently widely used in domestic animals, such as goats, pigs, cows, and a variety of tissue [12,22–25]. RNA-seq studies can rapidly and comprehensively examine transcribed genomic elements hidden in the genome and help researchers discover novel protein-coding and non-coding transcripts, which are more powerful compared with traditional methods based on public sequence information [26,27]. Therefore, in past ten years, many researches have focused on the molecular mechanisms of hair follicle development and cycling control in human [8,28–30]. More and more evidences show that hair follicle transformation during cycling is caused by alterations in the local signaling milieu [29–32]. Further research demonstrated that Wnt/wingless and Sonic hedgehog (SHH) signaling pathway, transforming growth factor-β (TGF-β) signaling pathway, and fibroblast growth factor (FGF) and tumor necrosis factor (TNF), play important roles in hair follicle development [33–35]. The in vitro experiments result showed that Wnt protein makes β-catenin accumulation in cell, which controls hair follicle morphogenesis and stem cell differentiation in skin [33,36]. In addition, FGF, which plays an important role in MAP/microtubule affinity-regulating kinase (MARK) signaling pathway is also a key factor in hair follicle cell differentiation. Previous studies showed that the follicle anagen will extend if *FGF5* gene is lost [30].

An interesting phenomenon is that almost all of hair follicle development researches are focused on cashmere goat rather than milk goat as experiment material. For example, Geng (2013) analyzed the DEGs in cashmere between differential hair follicle development stages [2]. However, in that research, authors used cattle genome as the reference genome— although the coding sequences between goat and cattle are highly homologous (up to 95% for many genes), using the cattle database to match goat sequences can still be limited. There are groups working on the primary and secondary hair follicle development using mouse model [37,38], however the differences in molecular mechanisms between primary and secondary hair follicle cycling are still not clear.

As genes important to cashmere hair follicle development were identified in the previous research [2,12,38], in this study, therefore, we analyzed the genome-wide expression using RNA-seq to explore DEGs related to cashmere (Arbus) and milk goat hair follicle cycling in different seasons. In this study, we took the skin samples, which contained not only the hair follicles and also the subcutaneous adipose tissue, recently identified as critical for extrinsic modulation of hair follicle behavior [39,40]. It includes identifying genes expressed in a stage-specific manner, defining clusters of genes showing similar patterns of temporal expression, and identifying stage-specific candidate genes for additional functional analysis. The genes of the different expression clusters associated with different functional categories clearly indicate the molecular and cellular events involved in hair follicle development and cycling.

2. Methods

2.1. Ethics statement

The goat (include cashmere and milk goats) experiments were performed according to the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China; revised in June 2004) and approved by the ethics committee of Inner Mongolia Agricultural University.

2.2. Method of animal sacrifice

The animals were allowed access to feed and water ad libitum under normal conditions and were sacrificed humanely to minimize suffering.

2.3. Animal and sample preparation

Cashmere and milk goats were obtained from the Aerbasi White Cashmere Goat Breeding Farm (Inner Mongolia, China). A total of six 3-year old individuals (3 cashmere goats and 3 milk goats) were randomly selected and any two or more individuals with traceable phylogenetic relationship were avoided in the sampling process. In each goat, we obtained three pieces of skin samples as technical repeat in each hair follicle development stage (March, June, September and December). A total of 72 skin samples were obtained for this study, including 2 species, 4 developmental stages, 3 technical replicates and 3 biological replicates. The skin samples were collected according to the method provided in a previous study [2]. The feeding and management conditions are quite same for all the animals. 1 cm^2 skin samples were from the right mid-side of each sampled goat at four hair follicle developmental stages, and then frozen in liquid nitrogen and stored at −80 °C for further analysis. Approximately > 2000 secondary follicles in each skin samples.

2.4. RNA extraction and sequencing

Total RNA was treated with RQ1 DNase (Promega) to remove DNA. The quality and quantity of the purified RNA were determined by measuring the absorbance at A260/A280 and A260/A230 using smartspec plus (BioRad). RNA integrity was further verified by agarose gel electrophoresis. For each sample, 10 μg of total RNA was used for RNA-seq library preparation. Please be noted that equal amount of total RNA from technical repeats and biological repeats was pooled to maximize the library coverage of cashmere goat and milk goat skin mRNA. Polyadenylated mRNAs were purified and concentrated with

oligo (dT)-conjugated magnetic beads (Invitrogen) before being used for directional RNA-seq library preparation. Purified mRNAs were iron fragmented at 95 °C followed by end repair and 5′ adaptor ligation. Then reverse transcription was performed with RT primer harboring 3′ adaptor sequence and randomized hexamer. The cDNAs were purified and amplified, and PCR products corresponding to 200–500 bps were purified, quantified and stored at −80 °C until being used for sequencing.

For high-throughput sequencing, the libraries were prepared following the manufacturer's instructions and applied to illumina HiSeq 2000 system for 101 nt paired-end sequencing. We applied both Qubit and qPCR to check the quality and to quantify RNA-seq libraries.

2.5. RNA-seq raw data cleanness and alignment statistics

Raw reads were first discarded if containing > 2-N bases, then reads were processed by clipping adaptor, removing low quality bases, and discarding too-short reads (< 16 nt).

Cutadapt [41] (version 1.7.1) was used to get the clean reads. After that, clean reads were aligned to the *Capra hircus* genome (GCF_001704415.1_ARS1) and gene sequences (downloaded from National Center for Biotechnology Information (NCBI) [42] (https://www.ncbi.nlm.nih.gov/genome/?term=goat) by TopHat2 [43], Aligned reads with more than one genome location were discarded due to their ambiguous location. Uniquely localized reads were used to analysis the data quality by using dupRadar [44] with default parameters and to calculate the reads number and FPKM value (FPKM represents fragments per kilobase and per million) for each gene according to reads and genes genome location. FPKM facilitates the comparison of transcript levels between samples. Other statistical results, such as gene coverage and depth, reads distribution around start codon and stop codon, were also obtained.

2.6. Differentially Expressed Genes (DEGs) between two samples

After getting the expression level of all genes in all the samples, differentially expressed genes were analyzed by edgeR [45] with TMM normalization method [46]. EdgeR prefers data including biological replication. However, it allows the analysis of DEGs between samples without replicates, which requires a customized dispersion value. When we performed edgeR analysis of DEGs between transcriptomes in this study without sequencing replicates, the dispersion value was set as 0.03 according to the high similarity for all samples (PCC > 0.93). For each gene, the *p*-value was obtained based on the model of negative binomial distribution. The fold changes were also estimated within this package, and False Discovery Rate (FDR) was got by adjusting the *p*-value. 0.05 FDR and 1.5-fold change were set as the threshold to define DEGs. We then combined the DEGs in different groups to one DEG union set and did the following analysis.

Because our samples are set in a time series experiment, we also applied maSigPro [47] to analyze our data. We used the default threshold as 0.05 FDR and 0.7 R-squared.

2.7. Reactome, Gene Ontology (GO) and KEGG pathway functional analysis

Cluster analysis of gene expression patterns was performed with cluster software and Java Treeview software [48,49]. GO functional enrichment analysis was carried out using Blast2GO (version 2.5.0) (http://www.blast2go.org/). KEGG pathway analyses were performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) data base (http://www.kegg.jp). Reactome pathway analysis was performed online using reactome version V62 (http://reactome.org/) [50]. We chose Project to human in step 2 to get the final results, and removed symbols that the database does not recognize.

2.8. Immunohistochemical staining

The skin samples were obtained from four different cashmere goats at different hair follicle development stages. Skin biopsy sections were fixed with paraformaldehyde (America, Sigma pH 7.4) for a night at room temperature. They were then dehydrated with 70%, 80% and 100% alcohol for 1 h respectively. Next, we used xylene to make the organization transparent twice for about 1.5 h each time until the organization is transparent through light at 50 °C. Soaking with soft wax (Germany, Leica) and xylene mixture for 2 h, and then soak 2 h with soft wax. Finally, soak with geocerain in incubator (65–70 °C) overnight. The samples were cut into paraffin sections 5 μm thick using a microtome (Leica 2035, Germany) and dried at 65 °C. They were than dewaxed and back-watered with xylene and alcohol with the purity of 100%, 80%, 70% respectively. Wash with phosphate buffered saline (PBS) three times for antigen repair. IHC kits (Kit-9710) and 3, 3′-diaminobenzidine (DAB) kit (DAB-0031) were purchased from Xinmai Co., Ltd. (Fuzhou, China). The protocol of IHC experiments were performed by the kit manual. The antigen of FOXN1、FOXE1、Noggin and SFRP4 were purchased by Abcam Inc. (England), and the product numbers are ab113235, ab134129, ab16054 and ab154167 respectively. Slides were mounted with neutral balsam (Xinmai Co., Ltd. Fuzhou, China) and photographed with a microscope (Nikon 80i) connected to a digital camera system.

2.9. Validation of DEGs by qPCR and western blot

To confirm the differential expression of genes revealed by RNA-seq, four genes identified to be expressed differentially among different development stages were chosen randomly for qPCR validation. The primers designed for qPCR analysis were listed in Table S1 and actin was used as a reference control.

The qPCR was carried out on an ABI 7300 system (ABI, USA) using FastStart Universal SYBR Green Master (ROX) according to the manufacturer's instructions. The thermal cycling conditions used in the qRT-PCR were 95 °C for 10 min, followed by 40 cycling? of 95°Cfor 10s and 60 °C for 30s. The specificity of the SYBR green PCR signal was confirmed by melting curve analysis. There were three biological and technical replicates, respectively.

The March and December cashmere and milk goat skin samples were collected from Inner Mongolia Agricultural University. The skin samples were cut into 0.5 cm*0.5 cm square centimeters, put it into mortar and grind in liquid nitrogen. Sample powder was lysed for 30 min in ice-cold RIPA buffer, and then add 4 × SDS loading buffer and boil for 20 min. After that, centrifuge at 14,000 *g* for 5 min at room temperature. Supernatants were collected and stored at −80 °C with protein concentrations determined using a BCA (bisinchoninic acid) assay kit according to the manufacturer's instructions (Byotime, China). Protein samples (30 μg/well) and kaleidoscope molecular weight ladders were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for approximately 60-80 min at 120 V in the running buffer. Proteins were transferred to a PVDF (polyvinylidenefluoride) membrane in transfer buffer using a wet blotting method at 270 mA for 80 min. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline and 0.05% Tween 20 for 1 h at room temperature, and then incubated with primary antibody (the primary antibody of *Wnt2* was purchased from Proteintech Co. and the primary antibody of *Wnt11, Fgf10* and *TP53* were purchased from Bioss Co. The dilution ratio is 1:500) in TBST with 1% nonfat dry milk at 4 °C overnight. This was followed by three 10 min washes in TBST, a 45 min room temperature incubation with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (The dilution ratio is 1:10000) in TBST, and then another three 10 min washes in TBST.

Table 1
Obtain the high-quality clean reads.

Sample	Raw data	Clean reads	Percentage
C3	37,009,564	30,271,778	81.78%
C6	33,605,950	30,112,074	89.60%
C9	38,272,220	32,450,593	84.78%
C12	31,993,598	28,435,878	88.88%
M3	52,465,058	45,136,826	86.03%
M6	54,751,304	48,548,266	88.67%
M9	61,652,862	54,656,782	88.65%
M12	69,311,374	53,656,478	77.41%

3. Results

3.1. Massive Sequencing and Aligning to the Reference Genome

To maximize the coverage of cashmere goat and milk goat skin mRNA by RNA sequencing, eight RNA libraries covering four different hair follicle cycling stages/phases were constructed by pooling RNA isolated from three different individuals and three technical repeats for each of them. The four phases were represented by March, June, September and December. The cashmere goat RNA-seq libraries generated over 32 million raw reads from each library, and > 52 million raw reads from milk goat each RNA-seq library. After filtering, there are > 28 million clean reads in each cashmere goat RNA-seq library and over 45 million clean reads in each milk goat RNA-seq library. The percentage of cashmere goat clean reads in each RNA-seq library ranged from 82% to 89%, and the percentage of milk goat clean reads in each library ranged from 77% to 89% (Table 1).

Each season was represented by > 30 million reads in cashmere goat and > 50 million reads in milk goat, with the reads density being enough for the quantitative analysis of gene expression. In this study, > 78% of total reads from both cashmere and milk goat matched either to a unique or multiple genomic locations of each season (Fig. S1). We also analyzed the reads distribution on the reference genome, and the result showed that > 80% reads are distributed on CDS and intergenic areas (Fig. 1A). We used dupRadar [44] to evaluate our RNA-seq quality, showing PCR duplicates in the sequenced cDNA libraries were very low and indicating a good quality of the sequencing data (Fig. 1B).

3.2. Analysis of DEGs at different seasons in cashmere and milk goat

Global sample correlation analysis revealed that the four cashmere goat samples were more similar than the four milk goat samples and clustered together (Fig. 2A). To identify the DEGs of hair follicle cycling in different seasons in cashmere and milk goats, we firstly analyzed the gene expression patterns using the edgeR [45] (FC ≥ 1.5 and FDR < 0.05). During the two different months, in cashmere goat, a total of 565 DEGs were found in all different combination. Between March and June, 169 DEGs were found, including 42 upregulated and 127 downregulated genes (Fig. 2B, Table S2). Between March and September, 285 DEGs were found, including 94 upregulated and 191 downregulated genes. Between September and June 84 DEGs were found, including 58 upregulated and 26 downregulated genes. Between December and March, we found that 232 genes were expressed differentially including 144 upregulated and 88 downregulated. Between June and December, 209 genes were differentially expressed, including 170 upregulated and 39 downregulated genes. Moreover, Dec. vs Sept. revealed only 69 DEGs including 50 upregulated and 19 downregulated genes.

However, 2860 DEGs were found in milk goat in each of the two months. A total of 295 DEGs were found between June and March, with 25 upregulated and 270 downregulated (Fig. 2C, Table S2). Between September and March 1658 genes were differentially expressed, including 793 upregulated and 865 downregulated genes. Comparing September and June, 259 DEGs were found, with 175 upregulated and 84 downregulated. Between December and March, 485 DEGs were found including 298 upregulated and 187 downregulated genes. Between June and December, 921 genes were differentially expressed, including 568 upregulated and 353 downregulated genes. After a comparison between December and September, there were 1715 DEGs, with 1009 upregulated and 706 downregulated genes.

The results showed that in the cashmere goat, the differentiation of expressed genes between Mar. and Dec. is the largest than other comparisons, while a relatively smaller differentiation arises between Sept. and Dec (Fig. 2B). However, in milk goat, the result showed that the largest comparison of DEGs was between Sep and Dec and the smallest differentiation arises between Jun and Sep (Fig. 2C).

We also used a time-series analysis tool maSigPro [47] to analyze DEGs during the hair follicle cycling process, resulting in 2313 and 1834 DEGs in cashmere goat and milk goat, respectively. Among these DEGs, only 252 were overlapped DEGs between these two goat species (Supplementary Table S3). We then analyzed the overlapped DEGs

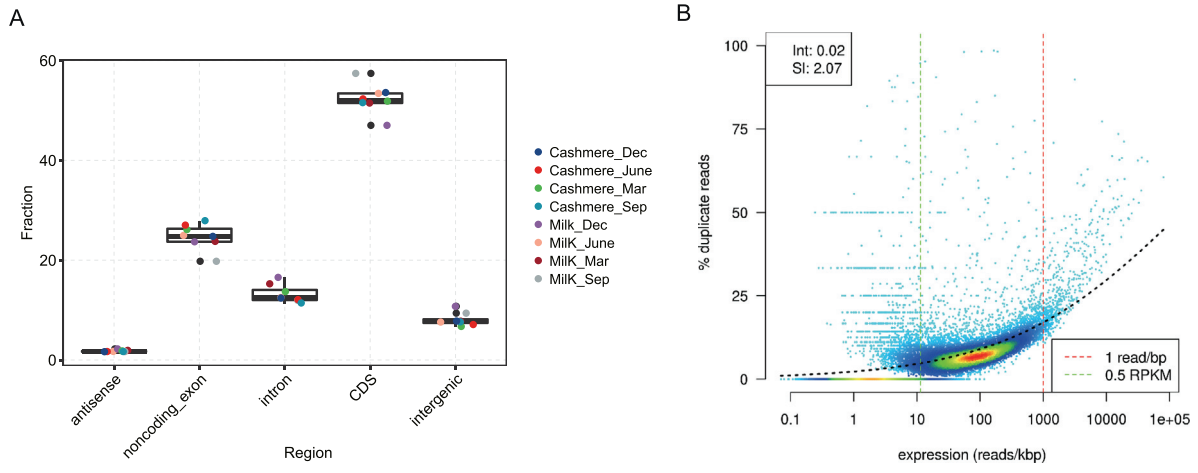

Fig. 1. Basic RNA-seq quality analysis. (A) Barplot of the mapping distribution of sequence reads across different genomic regions of the goat genome. (B) The relationship between expression and duplication level analyzed by dupRadar. The fraction of the duplicate reads was plotted against the expression level (reads/kbp) of each gene. The intercept and slope are obtained when fitting a logit model, showing that most genes are detected within a low percent of duplicate reads. The figure shown reflects the sequencing data from Cashmere goat living in December.

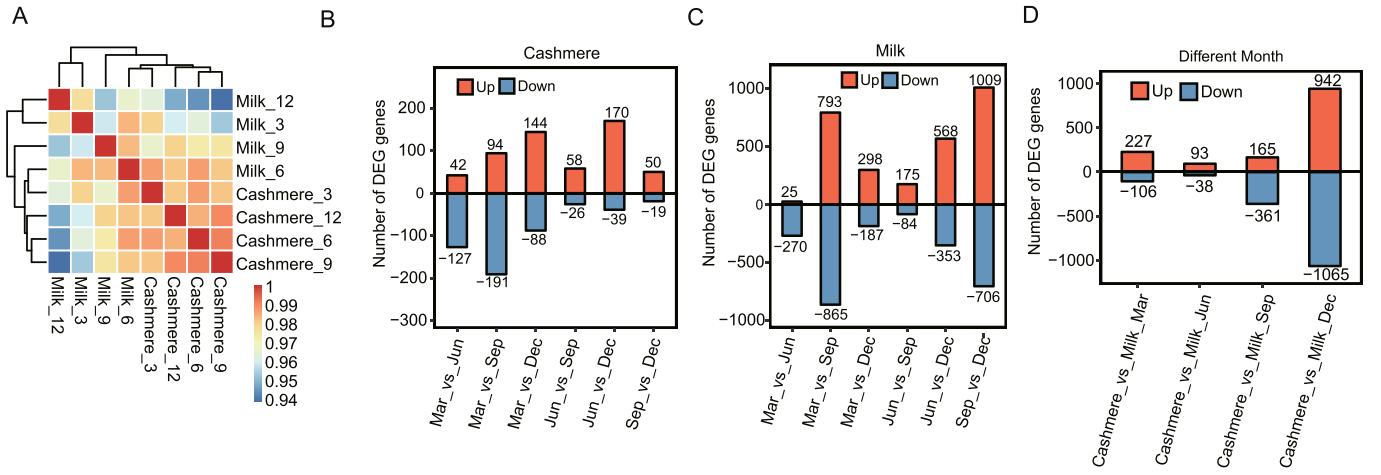

Fig. 2. DEGs controlled by seasons or goat genetics. (A) Heatmap showing the sample correlation analysis of all the eight sequqnced samples. DEGs regulated by seasons in cashmere goat (B) and milk goat (C), as well as those regulated by the genetic difference between milk and cashmere goat (D) were analyzed and plotted.

between edgeR and maSigPro. This analysis results revealed that 226 and 101 DEGs were both detected by these two methods in cashmere goat and milk goat, respectively (Fig. S2), indicating the substantial difference of these two methods when calling DEGs. Taken together, our results may suggest that there are considerable differences of physiological processes at different months of hair follicle cycling between cashmere goat and milk goat.

3.3. DEGs analysis between cashmere and milk goat

We then compared the gene expression patterns between cashmere and milk goat by using the edgeR [45] (FC ≥ 1.5, FDR < 0.05). The result showed that a total of 2399 DEGs were found between cashmere goat and milk goat (Fig. 2D, Supplementary Table S4). In March, a total of 333 genes were differentially expressed between Cashmere and milk goats, with 227 upregulated and 106 downregulated genes. In June, 131 genes were differentially expressed between Cashmere and milk goat, with 93 genes upregulated and 38 downregulated genes. In September, 526 genes were differentially expressed, with 165 upregulated and 361 downregulated genes. When we compared the gene expression pattern between Cashmere and milk goat in December, we found 2007 DEGs, with 942 upregulated and 1065 downregulated genes.

3.4. Functional classification analysis in cashmere and milk goat

Both GO and KEGG analyses were applied to understand the functional clustering patterns of DEGs between different seasons of either cashmere and milk goats obtained using edgeR. DEGs are enriched in important GO terms related to follicle hair cycling, such as the collagen fibril organization, hair follicle morphogenesis, keratin filament, as well as signaling (Fig. 3). DEGs related to follicle hair cycling were found, such as Wnt, TGF-b, Notch, Hedghog and MAPK signaling pathways in KEGG or GO terms (Table 2), but not significantly enriched. Both GO and KEGG analysis revealed substantial differences between the milk goat and cashmere goat. For example, the regulation of collagen fibril organization was pronounced between June and September for cashmere but not for milk goats. In general, more pronounced regulation of the follicle hair recycling occurs between March and December in milk goat, but between June and December or September and December in cashmere goat.

To further understand the hair follicle cycling mechanisms, we carried out functional classification analysis for DEGs obtained by maSigPro from cashmere goat and milk goat using Reactome Pathway Database [50]. Different from the standard DEG analysis tool, time-series analysis by maSigPro selects the genes whose expression patterns followed the time-series patterns. Consistently, heatmap analysis of maSigPro DEGs from this study showed an overall similarity in their expression patterns in both milk and cashmere goats. Clearly, genes showing better expression in March and June were selected (Fig. 4A and B).

In the cashmere goat, DEGs were enriched in multiple pathways associated with hair follicle cycling, which included “Hedgehog ‘on’ state”, “MAPK1/MAPK3 signaling”, “MAP2K and MAPK activation”, “Signaling by WNT”, “Signaling by NOTCH3”, “Signaling by NOTCH”, “Signaling by Hedgehog”, “Signaling by TGF-beta Receptor Complex”, “Formation of the cornified envelope”, “Signaling by BMP”, “Collagen formation” (Fig. 4D). A total of 128 DEGs were enriched in these hair follicle cycling-related functional pathways.

Meanwhile, the pathways which are important for hair follicle cycling were also enriched in season-regulated genes from milk goat, including “Assembly of collagen fibrils and other multimeri structures”, “signaling by TGF-beta family members”, “signaling by TGF-beta Receptor Complex”, “Collagen formation”, “Signaling by WNT”, “Signaling by BMP”, “Signaling by NOTCH”, “Signaling by NOTCH1”, “MAP2K and MAPK activation”, “Signaling by Hedgehog”, “Formation of the cornified envelope” (Fig. 4B). DEGs enriched in these functional pathways were 114. Interestingly, only 19 overlapped DEGs were identified, indicating that the molecular mechanisms of the hair follicle cycling vary in cashmere goat and milk goat. Furthermore, the top 11-enriched Reactome terms showed significant difference between these two types of goat (Fig. 4E and F, Table S5).

3.5. Functional classification analysis between cashmere and milk goat

In order to understand the DEGs in hair follicle cycling between cashmere and milk goat, we compared the same time DEGs between cashmere goat and milk goat using Reactome analysis. In March, 49 functional terms were identified ($P < .05$) (Fig. 5A, Table S6). Multiple DEGs were enriched in the pathways associated with hair follicle cycling, including “Formation of the cornified envelope”, “Keratinization”, “Crosslinking of collagen fibrils”, “Assembly of collagen fibrils and other multimeric structures”, “Anchoring fibril formation”.

In June, 51 functional terms were identified successfully ($P < .05$) (Fig. 5B, Table S6) “Collagen chain trimerization”, “Assembly of collagen fibrils and other multimeric structures”, “Collagen degradation”, “Collagen biosynthesis and modifying enzymes”, “Collagen formation”, “Anchoring fibril formation”, “Platelet Adhesion to exposed collagen”, “Formation of the cornified envelope”, “Crosslinking of collagen fibrils”, “Keratinization”.

In September, 81 functional terms were identified successfully

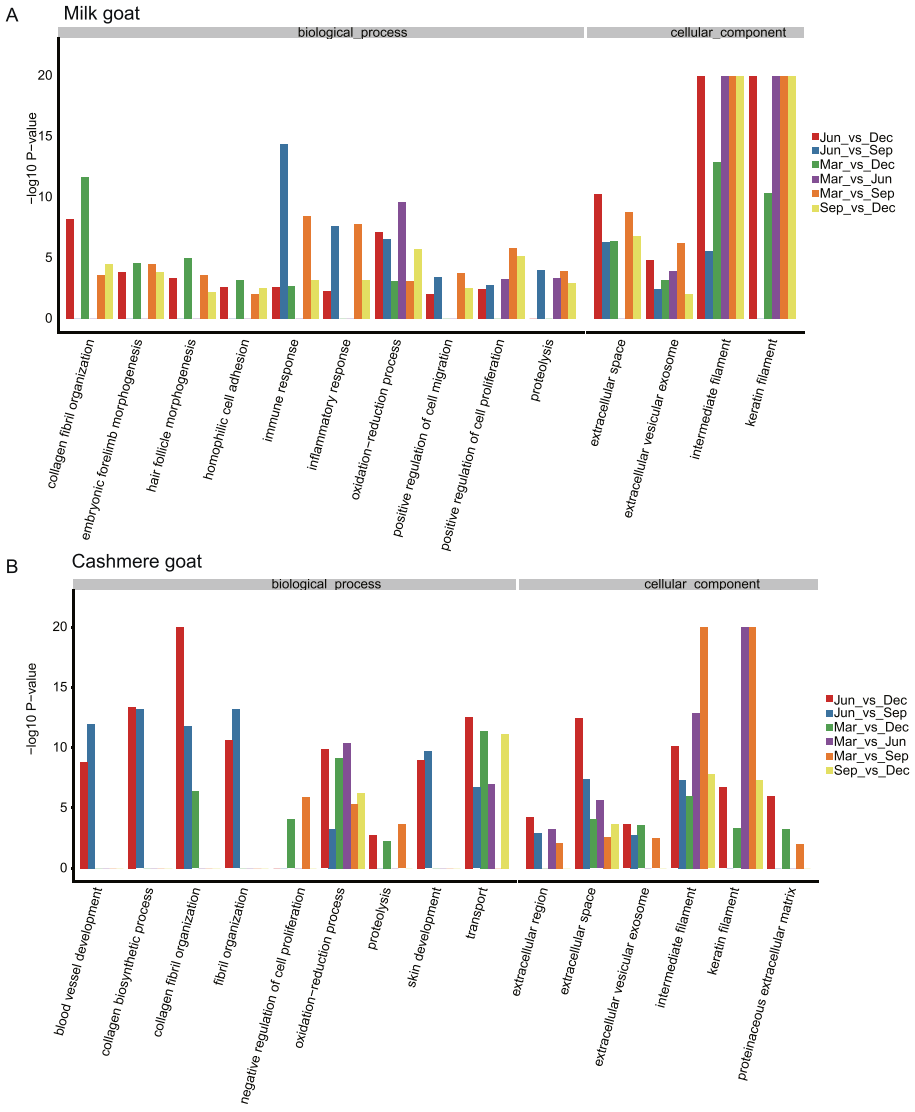

Fig. 3. GO analysis of differentially expressed genes (DEGs) of milk goat (A) and or cashmere goat (B) living in four different month (March, June, September and September). Each color represents a pair of compared seasons.

(P < .05) (Fig. 5C, Table S6), including “Collagen degradation”, “Formation of the cornified envelope”, “Keratinization”, “RUNX1 regulates transcription of genes involved in WNT signaling”, “Cilium Assembly”, “Hedgehog ‘off’ state”.

In December, 142 functional terms were identified sucessfully (P < .05) (Fig. 5D, Table S6), including “Formation of the cornified envelope”, “Keratinization”, “Assembly of collagen fibrils and other multimeric structures”, “Collagen formation”, “Collagen chain trimerization”, “Collagen degradation”, “Collagen biosynthesis and modifying enzymes”, “Pre-NOTCH transcription and translation”, “Beta-catenin independent WNT signaling.

3.6. The dynamic expression pattern of TGF-β/BMP, Wnt/β-catenin and FGF genes

To better understand the expression dynamics of the known regulatory genes of hair follicle cycling, we extracted all the isoforms of TGF-β/BMP, Wnt/β-catenin and FGF genes and had their expression level plotted. Fig. 6 and Table S7 showed that these genes are highly regulated in different seasons, which differed between cashmere goat and milk goat.

3.7. Localization of SFRP4, FOXN1, FOXE1, and NOG during different stages of hair follicle cycling in cashmere goats

As we have shown above, expression of many genes in hair follicle signaling pathways were more stable and altered to a lesser extent in cashmere goat than in milk goat. We selected some highly conserved such genes to probe their expression and localization at protein levels in cashmere goat skin from three different seasons using immunohistochemistry methods. *FOXN1*, *FOXE1*, *NOG* and *SFRP4* are very important genes in hair follicle development pathways in our study (Table 2) [51–54]. In this study, we have successfully determined the expression sites of FOXN1, FOXE1, NOG and SFRP4 in the skin of cashmere goats grown at different hair follicle cycling stages. The results showed that FOXN1, FOXE1, NOG and SFRP4 were expressed in the outer root sheath of hair follicles (Fig. 7A). Surprisingly, FOXE1 was also expressed in epidermal cells (Fig. 7B). The results are consistent with RNA-seq results.

3.8. Confirmation of gene expression dynamics by qPCR and western blot

In this study, we selected some representative signaling pathway genes to validate their dynamic expression during hair cycling in the

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Table 2
List of signaling pathways and genes potentially involved in cashmere and milk goat hair follicle cycling based on KEGG and GO analysis.

Sample	Signaling pathway	DEGs	Annotated DEGs
Cashmere goat	Hedgehog	3	SHH,LRP2,PTCH2,
	Notch	1	DLL4,
	TGF-beta	1	BMP5,
	Wnt	2	FOSL1,SFRP2,
	MAPK signaling pathway	9	DUSP5,NR4A1,CACNA1I,RRAS,DUSP14,DUSP2,FGF22,PTPN5,FOS,
Milk goat	MAPK signaling pathway	40	TGFBR2,PTPN7,FGF5,MAPK12,MAP3K8,CACNA1I,HSPA2,STMN1,CACNA1G,FOS,BRAF,ELK4,NTRK2,PRKCB,CACNG2, PDGFRA,IL1B,MAP4K4,PTPN5,MAP3K13,RPS6KA2,RASGRP3,CACNA1B,PLA2G4A,FGF18,GADD45A,DUSP14,HSPA8, RAC2,MAP3K2,DUSP8,MEF2C,FGF21,PDGFRB,DUSP5,PLA2G4F,MECOM,DUSP2,FGF22,MAPK11,
	Wnt	25	FZD10,WNT16,LEF1,VANGL2,CAMK2A,BAMBI,WNT2B,DKK1,SFRP4,DAAM2,FZD4,NFATC1,RAC2,SFRP2,FZD3,PRKCB, NKD1,SOX17,WNT11,WNT2,MMP7,SFRP1,PLCB4,TCF7,CAMK2B,
	TGF-beta	11	TGFBR2,BMP5,BMP8B,BAMBI,SMAD7,RBL1,NOG,SMAD6,PPP2R1B,CDKN2B,BMP8A,
	Notch	3	DLL4,HDAC2,MAML2,
	Hedgehog	10	PTCH1,WNT16,WNT11,HHIP,GLI1,WNT2,WNT2B,DHH,SHH,PTCH2,
	keratinocyte differentiation	5	WNT16,DSG4,FOXN1,EPHA2,IVL,
	hair follicle morphogenesis	7	SNAI1,KRT25,FOXE1,SHH,KRT27,ATP7A,NGFR,
Cashmere goat vs. milk goat	MAPK signaling pathway	32	FGF5,MAPK12,DDIT3,CACNA1I,FOS,BRAF,ELK4,NTRK2,NTF3,CACNG2,CACNA1C,PTPRR,PLA2G4A,DUSP10,FGF18,IL1R2,DUSP14,RPS6KA1,RAC2,DUSP8,MAP3K2,MEF2C,FGF21,-DUSP5,NR4A1,MAPK8IP2,RRAS,MECOM,DUSP2,FGF22,DAXX,MAPK11,
	Wnt	23	FZD10,LEF1,WNT16,VANGL1,VANGL2,BAMBI,PRICKLE2,SFRP4,WIF1,ROCK1,RAC2,WNT5B,FZD3, NKD1,SOX17,WNT11,WNT2,MMP7,FZD6,DKK2,TCF7,PLCB1,CAMK2B,
	TGF-beta	13	CHRD,BMP5,BMP8B,BAMBI,RBL1,NOG,SMAD6,PPP2R1B,ROCK1,INHBA,CDKN2B,BMP8A,BMP6,
	Notch	3	MAML2,NOTCH1,CIR1,
	Hedgehog	7	WNT5B,PTCH1,WNT16,SHH,WNT11,PTCH2,WNT2,
	Keratinocyte differentiation	6	WNT16,DSG4,FOXN1,EPHA2,KRT10,IVL,
	Hair follicle morphogenesis	8	NOTCH1,KRT25,FOXE1,IGFBP5,KRT27,SHH,ATP7A,NGFR,

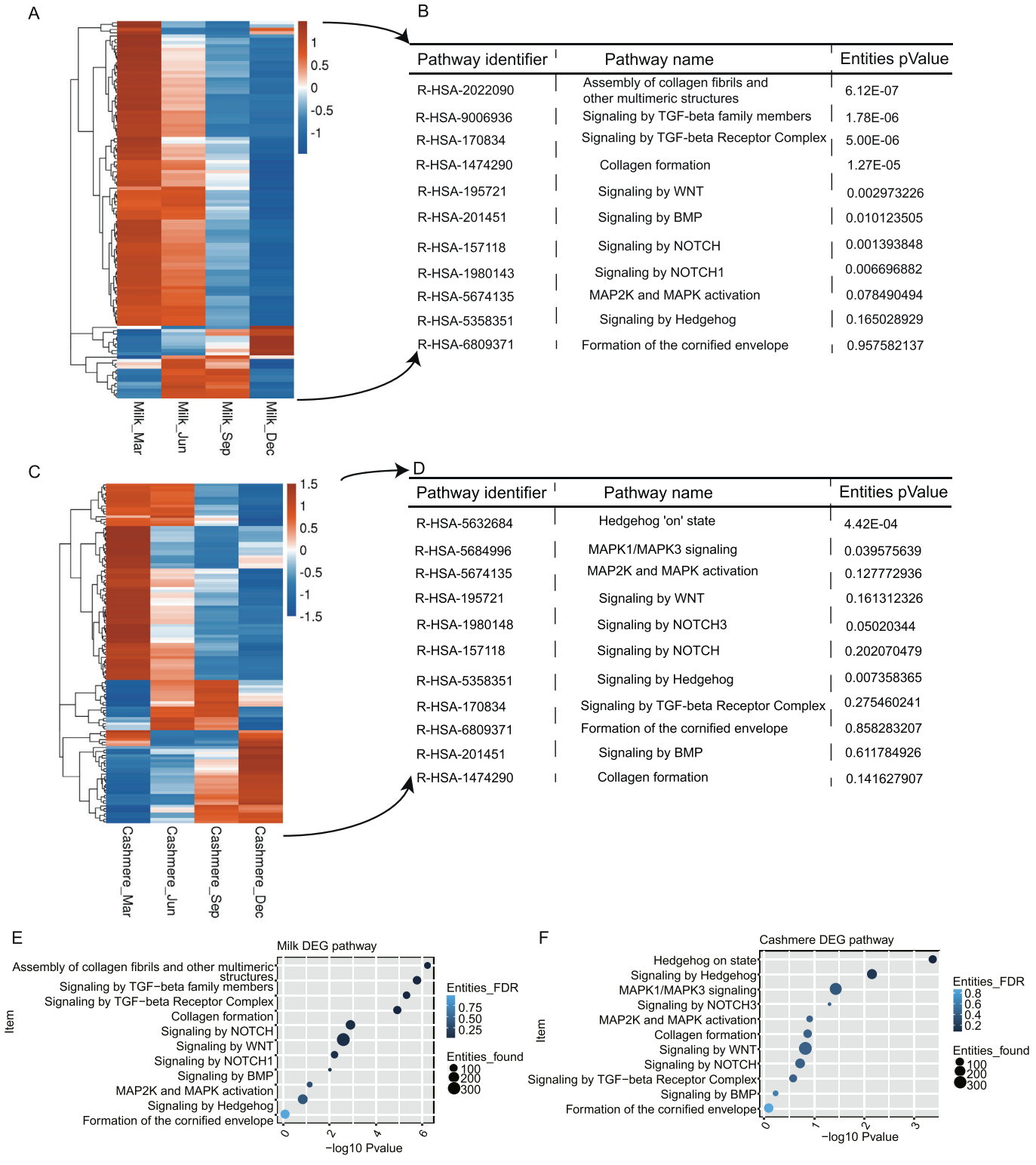

Fig. 4. Reactome analysis of DEGs by maSigPro method of milk goat (A-B, E) and or cashmere goat (C-D, F) living in four different seasons. The results for milk (A) and cashmere (C) Heatmap plotting the expression patterns of all DEGs. (B) and (D) Selected Reactome pathways enriched by DEGs and related to hair follicle cycling. (E) and (F) The top Reactome pathways enriched by DEGs.

milk and cashmere goats by both quantitative PCR and western blot. Among several genes that we selected, only anibodies against p53 and Wnt11 worked well. The results showed that *TP53* and *WNT11* were differentially expressed at different hair follicle development stages, and had the same trend with the RNA-seq findings (Fig. 8A and B). Thus, RNA-seq can provide reliable data for mRNA differential expression analysis from one side as with other similar findings.

4. Discussion

It is established that the existing hair follicles undergo cycles of growth (anagen), regression (catagen) and rest (telogen) to generate

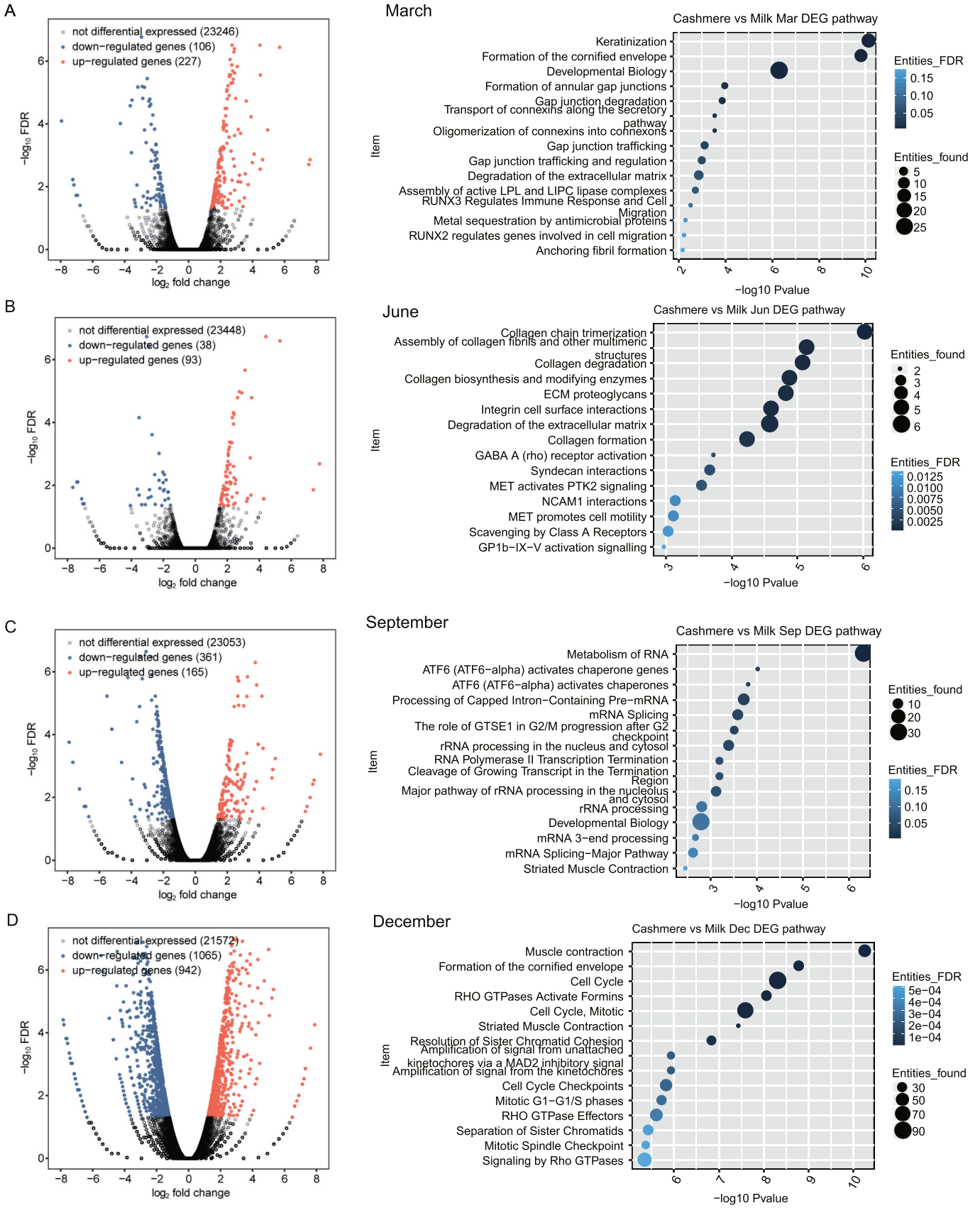

Fig. 5. DEG and their Reactome analysis between cashmere and milk goat at four different seasons. The left pannels show Volcano plots of DEGs and the right pannels show the top 15 Reactome pathways enriched by DEGs in March (A), June (B), September (C) and December (D).

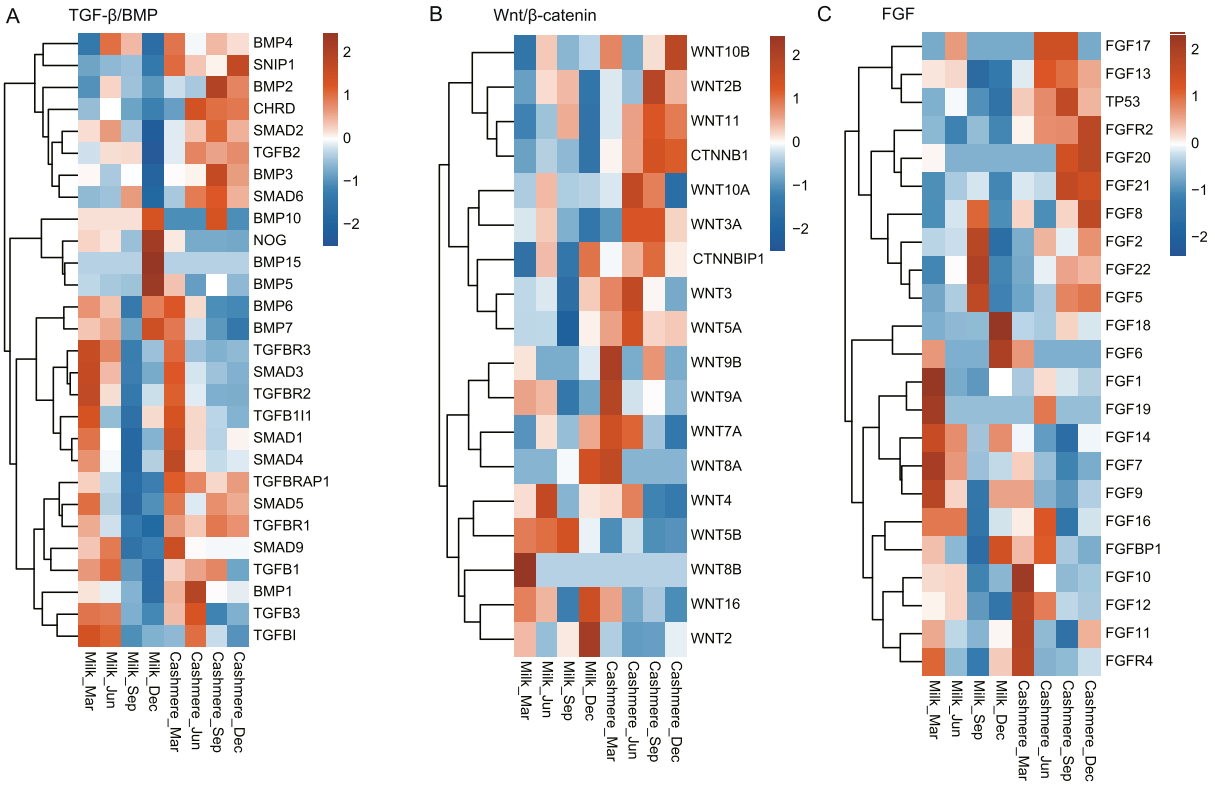

Fig. 6. Hierarchical cluster analysis of gene expression based on log ratio FPKM data. (A) All expressed TGF-β and BMP. (B) All expressed Wnt/β-catenin. (C) All expressed FGF. The color scale for upregulation and downregulation was shown.

new hairs each year for adult animals [55]. This study is the first research using RNA-seq method to compare the gene expression dynamics in skins from milk and cashmere goats, where only primary or both primary and secondary hair follicles are present, respectively, when the experimental goats were grown at four consecutive seasons of a full year. Comparison of skin transcriptomes of between seasons and between cashmere and milk goats in parallel demonstrated that expression of genes in the typical functional pathways for hair follicle cycling and development, which include WNT, NOTCH, MAPK, BMP, TGFβ and Hedgehog signaling pathways, were highly dynamic. Analysis of the differentially expressed genes (DEGs) demonstrated that the transcriptional switch is the largest between December and September, suggesting the transition from anagen to catagen occurs between September and December in milk goats. The exclusive expression of the resting *FGF18* in December suggested that the winter hair follicles of milk goats experience both catagen and telogen phases. In contrast, DEGs between December and September was the smallest in cashmere goats, and the season-regulated gene expression was much less than the milk, suggesting that the cycling mechanisms of secondary hair follicles in cashmere goats differ from the primary hair follicles.

4.1. Transcription in anagen, catagen and telogen phases of hair follicle cycle is distinct and season-regulated in adult milk goats

When hair grows in each anagen phase to produce the hair shaft from the tip to the root, the cell growth requires the activation of a number of cell growth signaling pathways, such as Wnt, Shh, TGFβ, EGF and FGF [55]. Consequently, the gene expression profiles in anagen phase are expected to differ from the neighboring catagen and telogen phases significantly. We found that DEGs between the consecutive months of March, June, September and December in milk goat samples were 25-up and 270-down (March to June), 175-up and 84-down (June to September), 1009-up and 706-down (September to December), 187-up and 298-down (December to March) (Fig. 2C). This large transcriptional shift suggests that the hair follicle transition from the anagen to catagen phase occurs when the milk goats enter the cold winter in the Mongolia region, China. The dynamic expression profiles of FGF5, TGFβ, BMP, and Wnt/β-catenin further supports the above conclusion (Fig. 6). We also noticed that the sequencing depth is not equivalent between cashmere and milk goat (Table 1). As the sequencing depth can influence the number of detected genes, as well as the expression level of detected genes. We proposed that sequence depth could be a possible factor for the difference of DEGs number.

Several lines of evidence show that FGF5, a member of the Fibroblast Growth Factor (FGF) gene family of intercellular signaling molecules, expresses in anagen and controls the transition from anagen to telogen [55]. A null mutation of the *Fgf5* gene leads to the angora phenotype which delays the transition between anagen and catagen [56]. *Fgf5* mRNA is expressed in the outer root sheath of mouse hair follicle during anagen, and anagen is abnormally prolonged in mice that lack exon 1 of the *Fgf5* gene. FGF5 receptor gene is expressed in the dermal papillae and seems to regulate the development and activity of the hair follicle [34]. Consistently, we found that expression of *FGF5* gene was the lowest in December (catagen) and highest in September (anagen) in milk goat, supporting the transition from anagen to catagen between fall and winter (Fig. 6).

In addition to FGF5, mammals have a large family of FGF members with various biological activities [57–72]. We found that the expression of *FGF2*, *FGF8* and *FGF22* showed the same dynamic pattern as *FGF5*, the highest in September and lowest in December. Expression of a large group of *FGF*genes was highest in March, and some in both March and June.

Fgf18 and Bmps are important factors for telogen maintenance in mice [73,74]. Consistently, we found that *FGF18* specifically expresses highly in December but low in other seasons, indicating that the hair follicles of milk goats are in catagen during December. Taken together the expression dynamics of *FGF5* and *FGF18*, we propose that the hair follicles of milk goat experience both catagen and telogen in winter, and

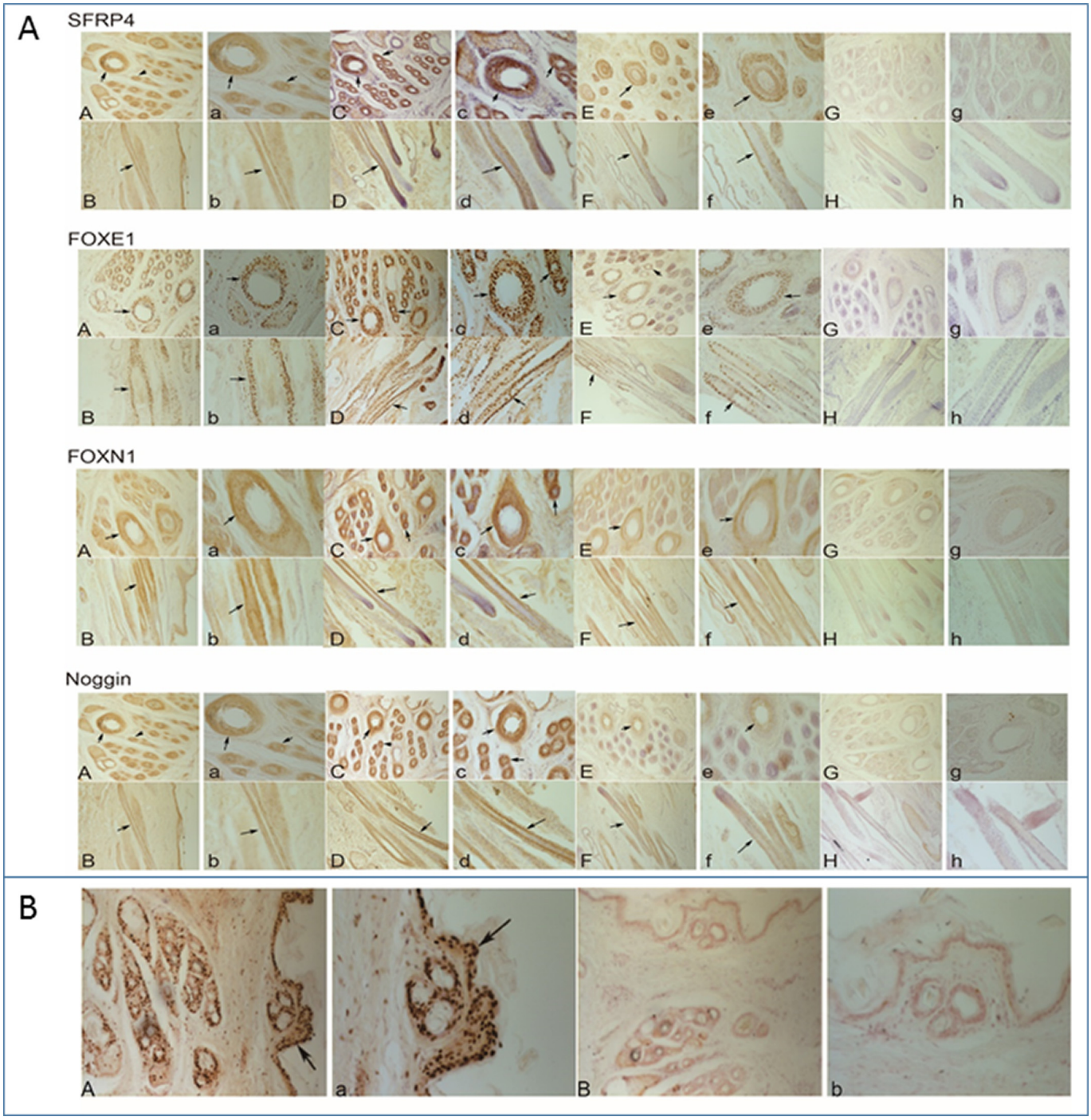

Fig. 7. The IHC results from cashmere goats grown in March, October and December. Panel A indicated the IHC results of SFRP4, FOXE1, FOX1 and Noggin in hair follicle. Panel B indicated the IHC results of FOXE1 in epidermal cell. In panel A, A–H indicated the IHC results with 100×, and a–h indicated the enlargement (200×). A, a, C, c, and E, e indicated the crosscutting results of hair follicle; B, b, D, d and F, f indicated the follicle slitting results of hair follicle. G, g and H, h indicated the negative control. In panel B, marked A and B indicated the IHC results with 100×, a and b indicated the enlargement (200×). A and a indicated the FOXE1 staining result while B and b for the negative control.

the anagen starts from March and ends in fall.

4.2. No anagen to catagen transition is observed in hair follicles when adult cashmere goats enter winter

Studies on cashmere goat hair follicles suggested considerable differences during the physiological process at different stages of cashmere goat hair follicle development [2,12,14,15]. Nevertheless, the difference between cashmere goat hair follicle development and the well-known primary hair follicle development characterized in mice remains obscure. In this study, a direct comparison of the transcriptome dynamics between cashmere and milk goats during the hair follicle recycling unexpectedly revealed the huge difference between these two different types of hair follicles. In contrast to the largest transcriptional transition from September to December in milk goats, the DEG number between these two seasons was the smallest in cashmere goats, strongly suggesting the lack of anagen to catagen transition between fall and winter (Fig. 2). This finding is further supported by the bulk expression

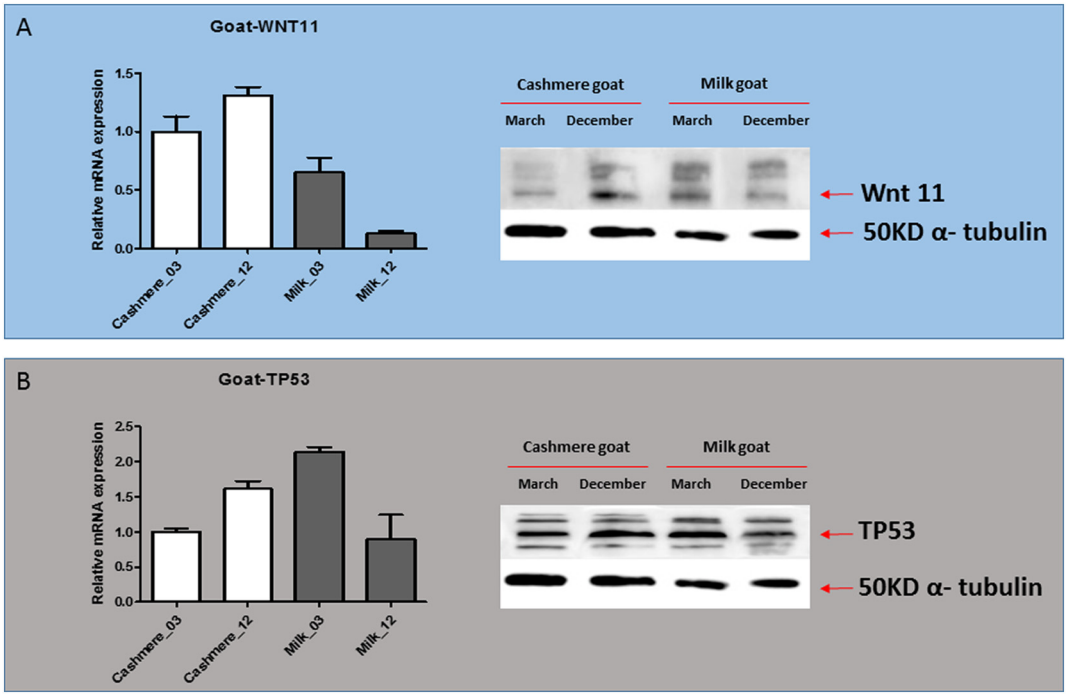

Fig. 8. Wnt11 and TP53 qPCR and western blot verification experiments results. White and black columns correspond to relative mRNA expression of cashmere goat and milk goat, respectively.

of *FGF5* in December similar as in September, and the high expression of the telogen *FGF18* in September. In addition to FGF5, the exclusive high expression of *FGF8* and *FGF22* in the same group in the September milk goats showed the concordant high expression in the September and December cashmere goats. In addition to the telogen *FGF18*, the anagen inhibitor TP53 [75] also showed highest expression in September (Fig. 6). Therefore, we concluded that cashmere goats do not experience catagen nor telogen in winter. The mixed high expression the anagen *FGF5,* telogen *FGF18*, and anagen inhibitor *TP53* in September and December together suggest mechanisms regulating the cycling of the secondary hair follicles differ significantly from the primary, which is worth of further studies.

We also analyzed the whole transcriptome similarity among all samples, revealing a much higher similarity between all cashmere goat follicle samples, but a larger difference among milk goat follicle samples. Additionally, maSigPro analysis revealed the similar number of DEGs with the same time-series expression patterns, however, Reactome analysis revealed the strong enrichment of DEGs in TGFβ signaling pathways in hair follicle samples of milk goats, but not in cashmere goats. In contrast, DEGs in cashmere hair follicles are enriched in Hedgehog “on” state, another essential for hair development [54,76–78]. All these results support the presence of different regulatory programs for hair follicle cycling in milk and cashmere goats.

4.3. The differential expression dynamics of FGF, TGFβ, BMP and Wnt/β-catenin in adult cashmere and milk goats

We obtained the expression dynamics of 19 *FGFs*, 2 receptors and one binding protein in the hair follicles of both milk and cashmere goats, showing four distinct expression groups (Fig. 6). The cashmere group are most well expressed in September and December, and some well expressed in June as well. TP53 belongs to this cashmere group. The milk group of *FGFs* were most well expressed in March and June. One shared group for March and June, another shared group for September milk, and September and December cashmere. The second shared group contains *FGF5*, *FGF8*, *FGF2* and *FGF22*.

Hair follicle regeneration begins when communication between quiescent epithelial stem cells and underlying mesenchymal dermal papillae generates sufficient activating cues to overcome repressive BMP signals from surrounding niche cells. BMPs belong to a superfamily that includes transforming growth factor β (TGT-β) [79]. TGF-β functions in tissue morphogenesis, homeostasis and cancer by regulating diverse biological processes including proliferation, apoptosis, differentiation, and extracellular matrix (ECM) production [80]. We obtained the expression dynamics of 9 *BMPs*, *TGFβ1–3* and 2 receptors, 7 *SMADs*. These classes of signaling proteins are also clustered into different groups. Similar as FGFs, most members in the major cashmere group are well expressed from June to December, which includes *BMP4*, *BMP3, BMP2, SMAD2, SMAD6, TFGβ2* (Fig. 6). Previous study showed that *Bmp2* and *Bmp4* are both activated in hair shaft precursors [81]. *Bmp2* is expressed specifically in the pre-cortex of the anagen hair follicle [82], while *Bmp4* and *Bmp6* are found to be expressed in the dermal papilla (DP) [82–84].

We found that more than a half of gene in BMP, TFGβ and SMAD families is well expressed in March in both cashmere and milk goats. Interestingly, the group containing *BMP5, BMP10, BMP15* and *NOG* shows a specific high expression in milk in December. Botchkarev and his colleagues proved that initiation of a new hair growth phase in postnatal skin requires neutralization of the inhibitory activity of BMP4 by the BMP antagonist noggin [85]. Previous studies showed that noggin inhibits TGF-β transduction [86]. Other studies showed that *noggin* and other neural inducing factors such as follistatin and chordin can inhibit TGF-β pathway in neural induction [87]. Our finding of specific high expression of NOG and a group of BMPs in December is consistent with the catagen and telogen phases in winter specific for milk goats.

Wnt genes encode short-range secreted signaling molecules that regulate cell fate, adhesion, shape, proliferation, differentiation and movement, and are required for the development of multiple organ system [88,89]. We and others have previously shown that several members of Wnt family, including *Wnt2*, *Wnt3*, *Wnt4*, *Wnt5a*, and *Wnt10a*, are expressed in the developing and mature skin and hair feather follicles [81,90–94]. In this study, we found two major clusters for the expression patterns of 16 *WNT* genes and β-catenin, the

cashmere goat and milk goat groups (Fig. 6). The cashmere group is large, and most members are well expressed in June and September. The milk group contains 5 members including *WNT2* and *WNT4* showing dominant expression in March. The third group showed dominant expression in cashmere in March.

5. Conclusions

Study of the mechanisms of hair follicle cycling in cashmere goat is important for the wool industry. Here we used comparative transcriptomic approach and revealed a large transcription program difference in the hair follicle cycling between the cashmere and milk goats. Our results suggest the lack of typical anagen, catagen and telogen phases for hair follicle cycling in cashmere goat, although they are present in milk goat. The cycling mechanisms of secondary hair follicles in cashmere goats apparently differ from the primary hair follicles in milk goats, which should be further studied. This study offers new gene expression information sources related to direct comparison of hair follicle cycling in milk and cashmere goats, which could be applicable to improve the wool production and quality.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2019.02.013.

Abbreviations

Not applicable.

Ethics approval and consent to participate

The goat (include cashmere and milk goats) experiments were performed according to the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China; revised in June 2004) and approved by the ethics committee of Inner Mongolia Agricultural University.

Consent for publication

Not Applicable.

Availability of data and material

Complete RNA-seq data sets can be accessed through GEO via series GSE84947.

Competing interests

We declare that none of the authors have any competing interests.

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Authors' contributions

YJZ, JQL and YZ designed and managed the project. ZYW, WJH and LLW prepared the samples and performed the experiments. RS, RJW, ZHL, YHZ, ZXW participated in some parts of the projects. KJW, DC and YXW analyzed the data and generated the graphics. YZ, YJZ, LLW, LLZ and JQL wrote and/or provided constructed ideas for the paper.

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