



Screening and Identification of Genes Related to Fibroblast Growth Factor 5 Hair Growth Regulation of the Liaoning cashmere Goat

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ABSTRACT

This study was conducted to screen genes related to hair growth of the Liaoning cashmere goats with fibroblast growth factor 5 (FGF5) treatments and study the molecular regulatory mechanisms of cashmere growth. FGF5 of different concentrations was used to treat skin fibroblasts of the Liaoning cashmere goats over several periods, and the total RNA of the fibroblasts was extracted. Subsequently, the expressed genes were screened by high-throughput sequencing, and the target genes were verified by qPCR. RNA-seq analysis showed that in the F4_24H, F4_72H, and F6_24H groups, 164, 189 and 123 transcripts were expressed in different amounts compared with the control group (group C). qPCR verification showed that the expression levels of OPN and ITGAV genes were up-regulated, which was consistent with the gene-sequencing analysis results obtained from F4_24H vs C group. The CCK-8 results showed that cell proliferation was the most pronounced in the F4_24H group. Therefore, FGF5 is verified to promote the proliferation of skin fibroblasts of Liaoning cashmere goats. Besides, in the F4_24H group, the OPN gene and its downstream ITGAV gene were demonstrated to enhance villus growth by regulating the ECM signaling pathway.

Keywords: Liaoning cashmere goat, fibroblast growth factor 5, signaling pathway, transcript

1. INTRODUCTION

Liaoning cashmere goat is a renowned Chinese goat species that produces cashmere of high values and excellent quality, which is known as the “fiber gem” in the textile industry. Thanks to their genetic variability, the goats are highly adaptable to various external environments, and it becomes valuable to study the molecular mechanisms behind cashmere production and quality [1]. In general, cashmere fibers are generated by the secondary hair follicle. The anagen phase is the active phase of cashmere fiber where major changes in the

amounts of gene expression in the secondary hair follicles occur [2]. The hair follicle is a vital structure for cashmere growth, and its growth and development are regulated by many hormones and cytokines (e.g FGF5 that affects villi growth) [3]. Specifically, FGF5 is a crucial participant in the periodicity of hair growth in mammals and a key signaling molecule to instruct hair growth to regression. FGF5 accumulates around the dermal papilla during long-term development. He et al. found that FGF5 is expressed in primary

and secondary hair follicle dermal papilla cells of cashmere goats [4]. Besides, FGF5 is also an important molecule in villi growth and the development of hair follicles, and its effects vary at different points during the growth of hair follicles. Moreover, previous studies have revealed that the expression levels of FGF5 vary across different stages of goat skin tissue development, and FGF5 predominantly affects hair length and the periodic activity of hair follicles [5,6].

The formation and periodic development of hair follicles are molecular processes controlled by multiple signaling pathways. Specifically, the PI3K-Akt, ECM, and FAK signaling pathways are involved in villus growth, and the PI3K/Akt/mTOR signaling pathway is vital for cell growth and metabolism of the hair [7]. Particularly, Akt is activated by growth factors and cytokines via the PI3K pathway, and ERK, PI3K, GSK-3 β , and β -catenin, whose expression and activation are associated with the Akt signaling pathway, are significant factors for hair growth [8]. Akt activation in Lgr5⁺ cells promotes hair regeneration. Moreover, Wang et al. found that Akt/ β -catenin signaling in macrophage-TNF-induced Lgr5⁺ hair follicle stem cells promotes the circulation and regeneration of folliculus pili after trauma [9]. Besides, ECM, which is capable to regulate cell and hair development, is the essential niche for hair follicle stem cells [10]. In hair follicles, raised stem cells produce ECM proteins that keep the HFSCs at their correct locations and functions by interacting with the α 8 β 1 integrin receptors in muscles [11]. FAK is an adhesion plaque complexes, as well as a non-receptor protein tyrosine kinase that is vital in the cytoplasm. Recent studies have shown that FAK can regulate cell growth, proliferation, migration, malignancy, and apoptosis. Ridgway et al. proposed that, the specificity of the skin loss of FAK causes a decrease in the activity of stem cell metabolism in the bulge region of hair follicles [12].

RNA-Seq technology is a high-throughput sequencing technique that detects transcripts even

with a low abundance. Besides, it can identify new transcription units, and reveal expression differences among samples. At present, RNA-Seq has been widely used in agricultural animals, such as sheep, cattle, and pigs [13,14,15]. Kang et al. analyzed the transcriptome of Chinese Tan sheep skin tissues by RNA-Seq and found that KRT25, KRT5, KRT71, and KRT14 genes are involved in hair formation [16]. Previous studies have shown that several genes may regulate cashmere growth together [2]. However, few scholars have studied how FGF5 influences villus growth and affects the expression of relevant genes in the skin fibroblasts of Liaoning cashmere goats with RNA-Seq. In this study, following the RNA-Seq analysis, the transcripts were screened for those relevant to the growth of Liaoning cashmere goats by GO and KEGG enrichment analyses and qPCR. This study is of reference value for future studies about the molecular regulatory mechanisms of cashmere growth.

2. MATERIALS AND METHODS

The study protocol was approved by the ethics committee of Liaoning Normal University.

2.1 Cell Culture and Drug Treatment

Experiment animals were obtained from the Liaoning Provincial Cashmere Goat Breeding Center. Five adult male goats (about 2 years old) were randomly selected to extract skin fibroblasts. The groups and the corresponding treatment settings were as follows: F4_24H (10⁻⁴ g of FGF5 for 24 h), F6_24H (10⁻⁶ g of FGF5 for 24 h), and F4_72H (10⁻⁴ g of FGF5 for 72 h). The FGF5 doses in the experimental groups were dissolved by PBS, and the two control groups were added with the same amount of PBS solvent and cultured for 24h/72h, respectively.

2.2 RNA Extraction

RNA extraction was performed based on the RNAiso Plus protocol. Reagents were purchased from TaKaRa (Dalian) and the DNAs in the RNA

genome were removed following the TaKaRa (Dalian) DNase I (RNase Free) protocol. The RNA quality was assessed by electrophoresis.

2.3 RNA-Seq Library Sequencing and Quality Testing

Library sequencing was conducted on an Illumina HiSeq™ 2500 using the PE125 sequencing strategy. The raw reads were analyzed by fastx_toolkit (v0.0.14) and the quality of the bases was detected following the QPhred = $-10\log_{10}(e)$ formula using Illumina Casva v1.8.

2.4 Mapping

Clean readings were mapped to a reference genome by TopHat2 (V2.0.9). Subsequently, the differentially expressed mRNAs were screened, followed by enrichment analysis. mRNA screening was performed by Cuffdiff with P-adjust <0.05 and $\log_2 >1$ (fold change), and the screened transcripts were assigned to genes by GSeq and enrichment analyses with a corrected p threshold of <0.05. With KEGG analysis, data of the selected Liaoning cashmere goats were compared with those in the NCBI database to identify the genes with different expressions and those genes may be linked to specific cellular pathways.

2.5 q-PCR Verification of Differentially Expressed mRNA

The RNAs were extracted and purified by adding 1 µg of RNA and 10 µL of DEPC•H₂O, followed by reverse transcription into cDNA. The compound was shaken thoroughly to ensure homogenization and incubated at 65°C for 5 minutes before being immediately placed in the ice bath. Subsequently, 4.1 µL of 5× reaction buffer, 0.6 µL of RiboLock™ Ribonuclease inhibitor (20 U/µL), 2.1 µL of dNTP Mix (10 mM), 1.1 µL of Reverse Tra Ace, and 1.1 µL of Oligo (dT) primer (0.5 µg/µL) were added into the sample. The mixture was incubated at 42°C for 60 minutes and 72°C for 10 minutes. After that, the mixture would either undergo PCR immediately or be frozen at -20 °C for later use. Using the cDNA obtained from reverse transcription as the template, primers of OPN, ITGAV, ITGA4, and ITGA9 genes were used to obtain their corresponding genes and the housekeeping genes (ACTB). Table 1 shows the primer sequences. The sample was placed in an AXYGEN PCR8 tube for centrifugation before the real-time PCR(RT-PCR) in an Eppendorf Realplex instrument, and the results were expressed by the $2^{-\Delta\Delta CT}$ method.

Table 1. Primer sequences of Real-time PCR.

Gene	Primer name	Primer sequence (5'-->3')	Products
ACTB	ACTB-F	GATGGCTACTGCTGCGTCG	208bp
	ACTB-R	GGCATACAGGTCCTTTTCGG	
OPN	OPN-F	CCGACGATGCTAACCCTGAT	194bp
	OPN-R	GCGGAACCTCTTCGATTTTGA	
ITGAV	ITGAV-F	GCCCCAAGCAAACACTACCC	283bp
	ITGAV-R	TGTCCCGTCCTGAAGAAAGC	
ITGA4	ITGA4-F	AGGCGGTGATGTTTCTGTTG	249bp
	ITGA4-G	AGGTCCTGGCTGGATTCTTC	
ITGA9	ITGA9-F	CCGAGTTGTTTCGCCATATCGT	208bp
	ITGA9-R	GCTTTCCAAAGTTCGGAGTTCA	

2.6 CCK-8 Detection of Cell Proliferation After FGF5 Treatment

Before treatment with 10^{-4} g/L of FGF5 for 24-72h, the skin fibroblasts of the Liaoning cashmere goat were starved for 12h. Cell proliferation was then assessed with a CCK-8 kit.

3. RESULTS AND DISCUSSION

3.1 Total RNA in the Samples

The target RNA fragment (ACTB) was amplified by RT-PCR, and agarose gel electrophoresis was performed on the amplified product. The electrophoresis results (Figure 1) showed only one band at 208bp of the housekeeper gene without dispersion, indicating that the genomic DNA had no amplification band.

3.2 Quality Assessment of the Sequencing Data

Since some of the original sequences were of low quality, they were filtered to get clean reads and ensure the quality of information analysis. As is shown in Table 2, the base error rate (0.03-0.04) was within the basic range of fluctuation, suggesting the data can be used for subsequent analysis. The rates of Q20 and Q30 bases were higher than 90%, indicating that filtered data were of better quality.

3.3 Mapping

Eighty percent of the sequences generated in the experiment were found to be conserved (Figure 2). Besides, the sequencing results of multiple mappings were about 2%, suggesting

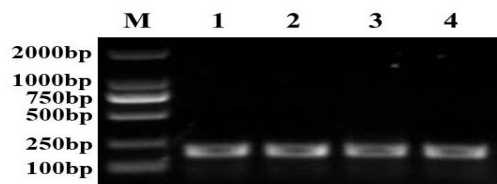


Figure 1. RT-PCR of housekeeping gene ACTB Lanes 1, 2, 3, and 4 represent F4_24H, F6_24H, F4_72H, and control group respectively

Table 2. Statistics of sequencing data.

Sample	Raw reads	Clean reads	Clean bases	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
C_1	48045300	46275212	5.78G	0.03	96.86	93.68	48.39
C_2	48045300	46275212	5.78G	0.04	94.99	90.67	48.37
F4_24H_1	51394058	49453178	6.18G	0.03	96.82	93.65	47.67
F4_24H_2	51394058	49453178	6.18G	0.03	95.31	91.23	47.57
F4_72H_1	49418892	47713121	5.96G	0.03	96.72	93.27	52.04
F4_72H_2	49418892	47713121	5.96G	0.04	94.71	90.09	52.26
F6_24H_1	46346088	44739804	5.59G	0.03	96.88	93.73	48.58
F6_24H_2	46346088	44739804	5.59G	0.04	94.92	90.55	48.55

C:control; F4_24H: 10^{-4} g/L FGF5, 24h; F4_72H: 10^{-4} g/L FGF5,72h; F6_24H: 10^{-6} g/L FGF5, 24h.

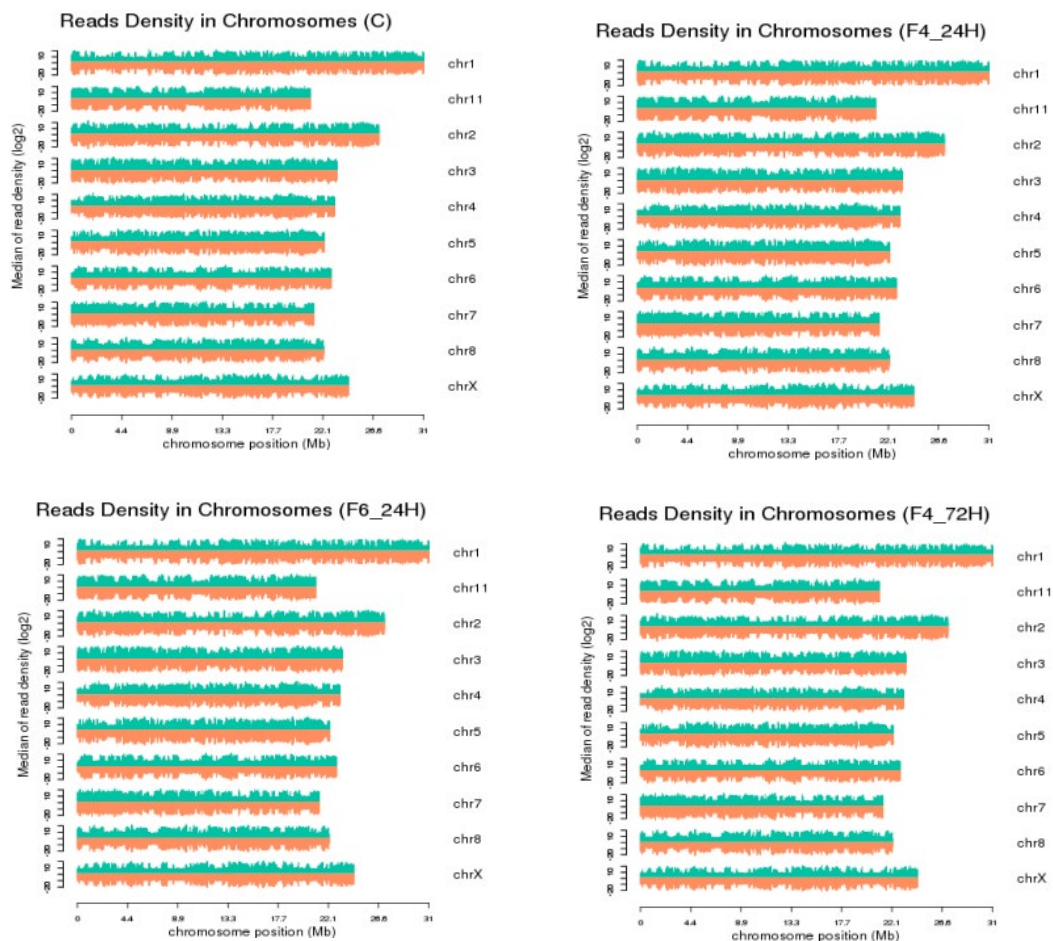


Figure 2. Density distribution of reads on chromosomes.

reliable experimental data with little contamination. The chromosome length was revealed to be positively correlated with the overall number of reads in the chromosome, and F4_24H, F6_24H, and F4_72H groups were more likely to map the goat chromosomes.

3.4 Analysis of Differentially Expressed Genes

In all experimental and control groups, the transcripts were screened and quantified by cuffdiff with a threshold $q < 0.05$ and $|\log_2\text{FoldChange}| > 1$. As is shown in Figure 3, compared to the C group, 164 transcripts are differentially expressed in

the F4_24H group, among which 70 were up-regulated and 94 were down-regulated. Similarly, 189 transcripts were observed to show differential expressions in the F4_72H group vs the C group, among which 78 were up-regulated and 111 were down-regulated. Moreover, 123 transcripts are expressed differentially in the F6_24H group vs the C group, among which 27 were up-regulated and 96 were down-regulated.

Figure 4 shows the hierarchical clustering analysis results, which confirmed the differential expression patterns of these transcripts under the experimental conditions. Three classification

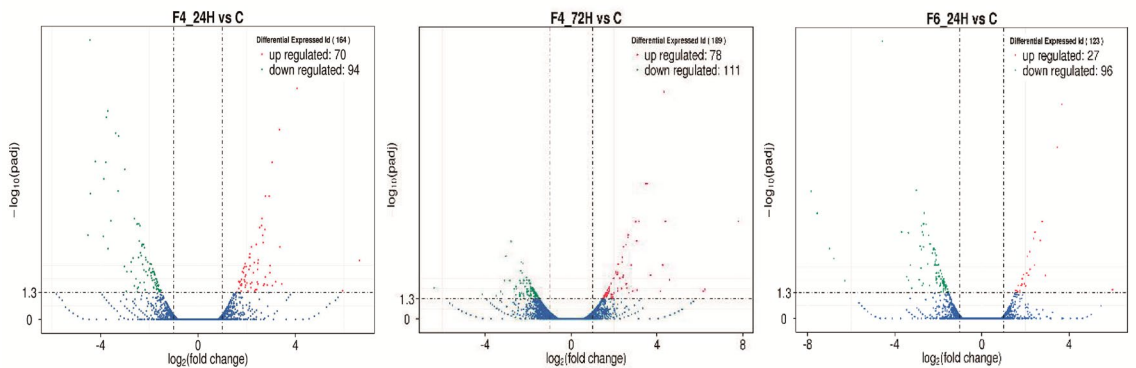


Figure 3. Differentially expressed transcript volcano map.

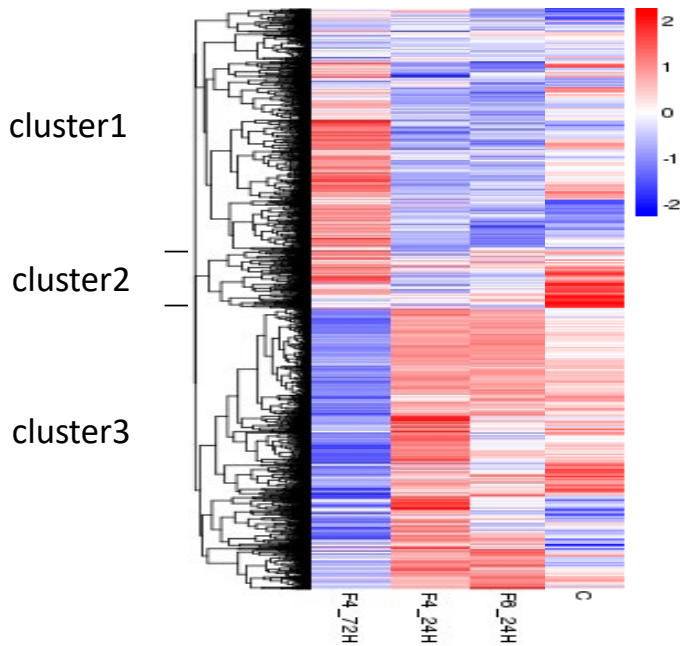


Figure 4. Differentially expressed mRNA clustering map.

clusters were identified. In Cluster 1, most genes in the control group exhibited low expression. Genes in the F6_24H and F4_24H groups revealed low expressions, while most genes in the F4_72H group were highly expressed. In Cluster 2, all the genes in the control group were highly expressed,

but the experimental groups showed similar gene expression patterns to those in Cluster 1. In Cluster 3, most genes in the control group were highly expressed, and the F6_24H group revealed similar patterns. Genes in the F4_24H group were all highly expressed, while all genes in the

F4_72H group showed quite the opposite pattern. In summary, in the F4_72H group, the expression levels of genes with differential expressions were the highest among all experimental groups.

The blue bar indicates that the FPKM value is less than 0, which is a minimally expressed transcript. The red bar indicates that the FPKM value is greater than 0, which is a highly expressed transcript

3.5 GO and KEGG Analysis of Differentially Expressed Genes

As is shown in Figure 5, according to the GO analysis results, compared to the C group, the screened genes ($P<0.05$) in the F4_24H group were predominantly and significantly enriched in terms of extracellular matrix in the cell composition (CC) and in the activities of metal endopeptidase and metal peptidase in terms of molecular functioning.

No abundant GO terminologies were observed in the F4_72H and F6_24H groups due to a lack of significant, functional enrichment (corrected $P<0.05$). Therefore, it can be inferred that functional enrichment is more dispersed.

The left axis represents the GO enrichment percentage of the specifically expressed gene and the right axis represents the number of genes enriched for each function.

As is shown in Table 3, KEGG enrichment analysis was performed at the level of signal pathways, with a screening threshold of $q<0.05$. Compared with the control group, 28 differentially expressed genes were observed in the F4_24H group, among which 8, 9, and 11 genes were significantly enriched in ECM-receptor interaction, focal adhesion, and the PI3K-Akt signaling pathway, respectively.

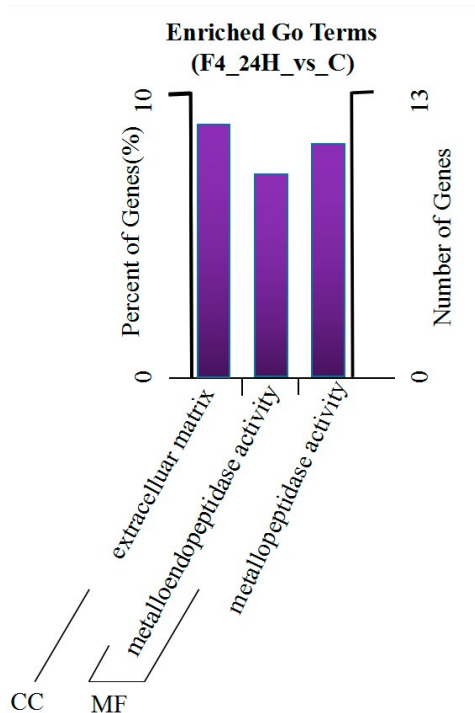


Figure 5. Gene ontology (GO) enrichment analysis chart.

Table 3. Signaling pathways and gene screened in F4_24H vs control group.

Term	Sample number (gene count)	Corrected P-Value	Genes	DiffExpr Analysis
ECM-receptor interaction	8	0.001161441	OPN(SPP1)	up
			ITGA6	down
			ITGB8	down
			THBS1	down
			ITGA4	down
			ITGA8	down
			LAMA2	down
			LAMA4	down
Focal adhesion	9	0.039331188	OPN(SPP1)	up
			ITGA6	down
			ITGB8	down
			THBS1	down
			ITGA4	down
			ITGA8	down
			LAMA2	down
			LAMA4	down
PI3K-Akt signaling pathway	11	0.048332088	PDGFD	down
			OPN(SPP1)	up
			ITGA6	down
			ITGB8	down
			THBS1	down
			ITGA4	down
			ITGA8	down
			LAMA2	down
			LAMA4	down
			PIK3AP1	up
			SGK1	down
			PDGFD	down

3.6 q-PCR Verification Analysis Results

Figure 6 shows that, the expression levels of the OPN and ITGAV genes were up-regulated, but the expression of the ITGA4 gene was down-regulated. Meanwhile, the ITGA9 gene revealed no significant changes in its expression.

3.7 CCK-8 Verification Analysis Results

Figure 7 shows the CCK-8 result chart. Compared with the control group, all experimental groups revealed greater numbers of cells, but the

highest cell proliferation rate was observed at 24h.

In this study, the differentially expressed transcripts in each experimental group were found to be significantly enriched in several signaling pathways. Specifically, the ECM, FAK, and PI3K-Akt signaling pathways screened in the F4_24H group are crucial for villus growth. ECM and other relevant integrins can activate signaling pathways related to cell adhesion and focal adhesion, thereby activating downstream signaling pathways involved in cell proliferation

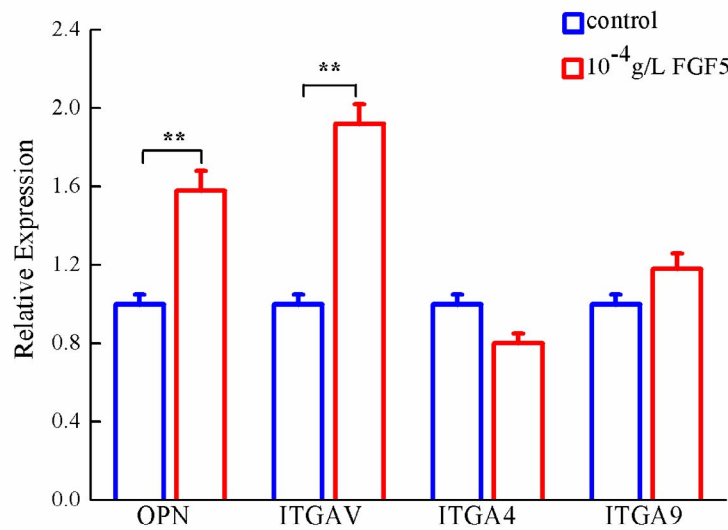


Figure 6. Real-time PCR validated expression levels of mRNA.

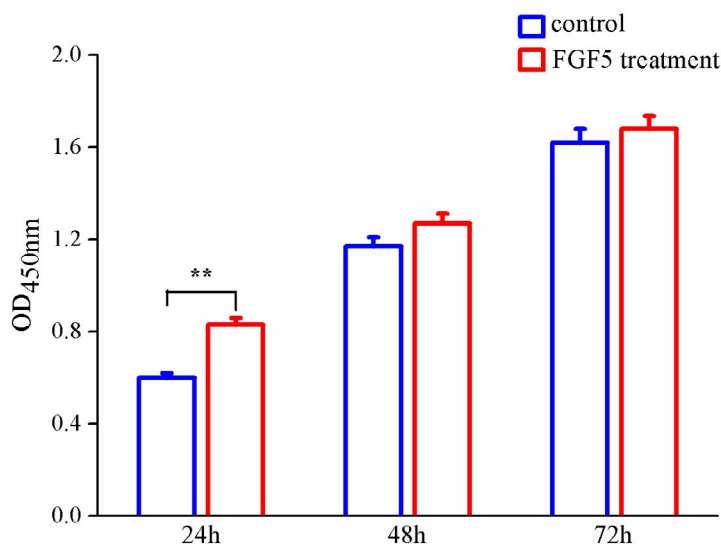


Figure 7. The OD value of Liaoning cashmere goat skin fibroblasts treated with FGF5 at different times.

and invasion. Moreover, the interactions between ECM and cell surface receptors could promote the growth of hair follicles [17]. FAK can integrate signals from multiple sources (e.g. ECM) and growth factors to control cell metabolism and proliferation. Furthermore, β -catenin also needs FAK to initiate stem cell proliferation in the bulge region of the hair follicles. Ridgway et al. found that FAK regulates TPA-induced β -catenin activation in the bulge region of hair follicles by preventing nuclear localization and transcriptional activation of key targets (e.g. c-Myc). In the skin, FAK is expressed only in the proliferative stromal area, outer root sheath, or the bulge region of hair follicles, and FAK expression is promoted in the regular hair cycle as a response to proliferation signals (e.g. TPA)[12]. The PI3K-Akt signaling pathway is also significant in cell proliferation[18]. Specifically, lncRNA5322 regulates the proliferation and differentiation of hair follicle stem cells by adjusting the miRNA-mediated PI3K/Akt signaling pathway [19]. Moreover, ginsenoside Rb1 promotes hair follicle growth by adjusting the PI3K/Akt/GSK-3 β signaling pathway and enhancing the proliferation and migration of dermal papilla cells [8].

In this study, the expressions of several genes from ECM, FAK, and the PI3K-Akt pathway, as well as the OPN gene, were up-regulated in ECM-receptor interaction, focal adhesion, and the PI3K-Akt signaling pathway. Moreover, from the ECM signal pathway map, it can be seen that the OPN gene is upstream of ITGAV, ITGA4, and ITGA9 genes, and changes in the OPN gene expression may directly affect the three downstream genes. The expressions of these four genes were verified by qPCR, and the results showed a significant up-regulation, which is consistent with the previous sequencing results. Particularly, OPN is an extracellular matrix (ECM) protein with multiple functions, and its overexpression in CAL-27 and SCC-25 cells can promote cell proliferation [20]. OPN expression is regulated by the transcription

factor NF- κ B, a vital factor to keep the human hair follicles at their growth phase [21]. In the early stages of hair growth, NF- κ B is highly active in the hair stromal epithelium, and scalp hair follicles proliferate rapidly. Key regulators of hair growth, such as TNF- α and IL-1, affect villus growth by adjusting the NF- κ B signaling pathway [22]. In a mouse model, the NF- κ B signal was detected in secondary hair follicles during the early growth of folliculus pili, suggesting that NF- κ B signaling factors may play a role in the anagen phase of hair follicle stem/progenitor cells. NF- κ B activity was also observed in the inner root sheath during the middle stage of hair growth, indicating the involvement of NF- κ B signaling molecules in hair morphogenesis [23]. In addition, ITGAV also participates NF- κ B signaling. A member of the integrin subunit alpha family, it is encoded by the ITGAV gene on chromosome 2 [24]. ITGAV inhibition would suppress the proliferation of stem cells in prostate and bladder cancers, which may be related to integrin-mediated NF- κ B signaling [25]. Moreover, ITGAV and ITGA5 regulate the proliferation of stem cells derived from human adipose and ITGAV shortage would induce cell death and inhibit cell proliferation [26]. Therefore, OPN and ITGAV may promote the proliferation of skin fibroblasts, thereby facilitating the growth of hair follicles.

4. CONCLUSIONS

FGF5 can promote the proliferation of dermal fibroblasts in Liaoning cashmere goats, and the cell proliferation rate is the fastest at an FGF5 dose of 10^{-4} g and an administration time of 24h. Three signaling pathways related to villus growth, namely ECM, FAK, and PI3K-Akt, as well as multiple genes showing differential expressions were screened out. Among them, OPN is found to affect villi growth by regulating the downstream target gene, ITGAV, thereby influencing the ECM signaling pathway.

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CONFLICT OF INTEREST STATEMENT

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

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