

10×Genomics Single-cell Sequencing Reveals Differential Cell Types in Skin Tissues of Liaoning Cashmere Goats and Key Genes Related Potentially to the Fineness of Cashmere Fiber

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Abstract

Cashmere fineness is one of the important factors determining cashmere quality, however, our understanding about the cells that make up the cashmere fineness is limited. Here, we used single-cell RNA sequencing (scRNA-seq) and computational models to identify 13 skin cell types in Liaoning Cashmere Goats. We also analyzed the molecular changes by Pseudo-Timeline Analysis in the development process and revealed the maturation process in gene expression profile in Liaoning Cashmere Goats. Weighted gene co-expression network analysis (WGCNA) explored hub genes in cell clusters related to cashmere formation. Secondary hair follicle dermal papilla cells (SDPCs) play an important role in the growth and density of cashmere. *ACTA2*, a marker gene of SDPCs, was selected for immunofluorescence (IF) and western blot (WB) verification. It was proved that it was mainly expressed in SDPCs, and WB results showed different expression levels. *COL1A1* is a high expression gene in SDPCs, which was verified by IF and WB. Then select the function gene *CXCL8* of SDPCs to verify, and prove the differential expression in the coarse type and the fine type of Liaoning Cashmere Goats. Therefore, *COL1A1* may involve in regulated skin development and *CXCL8* gene may regulate cashmere fineness. These genes may be involved in regulating the fineness of cashmere in goat secondary hair follicle dermal papilla cells, our research will provide new insights into the mechanism of cashmere growth and cashmere fineness regulation by cells.

Introduction

Cashmere goats are a kind of species with high economic value. Cashmere has a certain position in the international market, and the quality meets the requirements. Among them, the value of cashmere is the most concerned, but there is not much research on it. Cashmere includes two parts, primary hair follicle and secondary hair follicle, while the growth of cashmere is regulated by secondary hair follicles, and is the main source of cashmere¹. Cashmere goat hair grows from special hair follicles of the skin, and some genes related to hair growth get expressed in secondary hair follicles. Although the output of cashmere is high, the overall trend of cashmere is too coarse, affecting the formation of textile crafts.

We have detected genetics and backgrounds that affect cashmere fineness from different angles. Liu et al., have detected candidate genes closely related to cashmere fineness in Inner Mongolia cashmere goats by Genome-wide association analysis². *KAP* and its family are an important element that has been shown to regulate the fineness of cashmere³. In order to find a more reliable internal genetic background that determines the fineness of cashmere, we use the most advanced single-cell RNA-seq is a technique for observing gene expression at the single cell level⁴. Single-cell sequencing enables more precise selection of key genes involved in cashmere fineness in secondary hair follicle dermal papilla cells. Through it to determine the true determination of certain specific genes can be expressed in secondary hair follicle cells. In recent years, more and more studies have shown that single-cell sequencing can be used to reveal cell differentiation and localization, after rapid technological development, the latest single-cell RNA sequencing has been able to identify a large number of cells^{5,6,7,8}. In mice, small intestine single-cell sequencing was used to obtain helper cell types that support intestinal stem cells (ISCs) to produce mature epithelial cell types⁹. Nevertheless, single-cell transcriptomic reveals heterogeneous differentiation and spatial characteristics of mouse epidermis and hair follicles¹⁰. Furthermore, in order to determine the differences in oocyte maturation at different stages, transcriptome sequencing of single cell was compared oocyte maturation gene expression in children and female goats, and to screen and verify genes related to oocyte maturation¹¹. However, research on regulating the fineness of cashmere at the cellular level is still scarce.

Herein, the purpose of this study was to identify the differential cell types in the skin tissues of cashmere goats and to dig into the key genes associated with cashmere fiber fineness. Therefore, the cells of cashmere goat skins were sequenced using single-cell sequencing technology to systematically analyze the cell heterogeneity in tissue-stable epidermal cells. We employ pseudo-timeline analysis revealed molecular changes during development and demonstrated the maturation process in gene expression profiles. And the use of immunofluorescence (IF) and Western blot (WB) to verify new key genes may be a turning point in regulating the fineness of cashmere. Through the deep research and analysis of cashmere skin cells, more gene information can be obtained to screen the genes related to cashmere fiber fineness more clearly and accurately, so as to improve the quality of cashmere and increase the economic benefit, and lay the foundation for the future research on cashmere fiber fineness correlation.

Results And Analyses

The single-cell sequencing identifies 13 distinct cell populations in goat skins. To better define the cell types in Liaoning Cashmere Goat skins, we performed a single-cell RNA sequencing analysis in coarse type of Liaoning Cashmere Goat skins (CT-LCG) and fine type of Liaoning Cashmere Goat skins (FT-LCG). For each type goat, we obtained 551,655,085 and 556,307,136 reads. The sequencing saturation rates were 76.9% and 68.3%, respectively (Fig. 1a). We separated and sequenced 6,102 and 1,1197 cells, following strict quality control 3,443 and 9,608 cells were remained for downstream analysis in CT-LCG and FT-LCG.

We first defined different cell populations in cashmere goat skins, using the analysis of unsupervised clustering and t-distributed stochastic neighbor embedding dimension reduction (t-SNE). After standard quality control excluded cells including genes check <500 and mitochondrial genes coverage >10%, samples were detailed clustering analyzed by Seurat workflow definition 13 distinct population (Fig. 1b). The cells interaction between 13 clusters is shown in Fig. 1c. The average expression profile of the two samples is highly correlated, and the number of cells in each type is shown in Fig. 1d. We have identified the gene expression profile for per cluster, differential expression genes (DEGs) refer to the difference between the average expression of cells in clusters and that not in clusters (Fig. 1f). The top10 up expression genes of each cluster are shown in Table 1. In total, 2 kinds dermal papilla cells, 4 stem cells, 3 sheath cells, 1 keratinocyte, 1 fibroblast, 1 epidermal cell and 1 stromal cell identity was assigned in goat skin based on known cell type specific marker expression such as *CD34*, *KRT19*. We detected that genes enriched in distinct clusters differently marked various cell types. For instance, *KRT14* and *KRT5* are known expression markers in Epidermis stem cells (Cluster 1) (Fig. 1e). *EDAR* and *DKK1* are well known to be highly expressed in Epidermis cells (Cluster 2). Dermal sheath cells (Cluster 6) were identified with specific expression of known marker gene *BMP2*. Genes enriched in the Cluster 7 strongly labeled in Dermal stem cells and expressed cell markers such as *SOX2* and *FOXM1*. Hair follicle stem cells (Cluster 8, HFSCs) promote hair development, and were identified by multiple

specific markers (e.g., *KRT19*; *DSG3* and *ITGA6*). Skin stem cells (Cluster 12) also expressed selective genes, including *CD34* and *ITGB1*. Finally, we found that cells from C10 showed characteristic of Dermal papilla cells (DPCs), which is known to be high expression level of *CTNNA1*, and C11 showed characteristic of Secondary hair follicle dermal papilla cells (SDPCs) such as high expression levels of *VCAN* and *ACTA2*. Moreover, Dermal fibroblasts cells markers (e.g., *EGF* and *VEGFA*), Inner root sheath cells markers (e.g., *CDIP1*; *CDS1* and *FOXN1*), Outer root sheath cells markers (e.g., *DOCK6* and *KRT19*), Stromal cells marker (e.g., *ITGA6*) and Keratinocytes cells marker (e.g., *KRT6A*) showed specificity expression in Cluster 0, Cluster 3, Cluster 5, Cluster 4 and Cluster 9, respectively. In short, these results emerged the comprehensive transcriptional heterogeneity of cell types, which make up cashmere goat skins and revealed molecular generate markers to make further efforts study in diversities and functions.

Importantly, the high expression genes of per cell group are shown in (Fig. 1g). *KRT5* and *KRT14* are highest expressed in Epidermis stem cell, and significantly enriched in Cluster 0, Cluster 2, Cluster 7 and Cluster 8. *CCL27*, *ADIRF* and *LY6D* had obvious difference expression in Epidermis stem cells. *KRT10* are highest expressed in Cluster 4 and Cluster 5, *LOC102179515*, *NFIB*, *BGN* and *SFPRP1* was overexpressed on Cluster 8. Interestingly, *CXCL14* was overexpressed on Cluster 8 and Cluster 11, which may be related to hair development. Indeed, *KRTDAP* and *CST6* are highest expressed in Cluster 9. Notably, *KRT35* and *KRTAP11-1*, may be related to cashmere character¹², whereas in goat their expressions are confined to Dermal papilla cells. Additionally, *LEF1* is considered to be a hair-inducing gene of dermal papilla cells¹³. While the expression of the positive genes, *IGFBP5* and *IGFBP6*, are discovered highly expressed in SDPCs. *ALX4*, which is known may be related to cashmere fineness¹⁴, only expressed in DPCs and SDPCs. *CCL21*, *GNG11* and *TFPI2* are highest expressed in HFSCs, *VIM* and *CLEC3B* were overexpressed on DPCs and HFSCs. *KRT19* and *SOX9* were proven to exist in the anagen secondary HFSCs¹⁵, which have the high expressed in our results in HFSCs. Some genes are only expressed in certain cells, such as *PLVAP*, *PECAM1* and *CENPF* (Fig. 1h). These significant up expressed genes, especially over-expressed in each functional cluster, and may be functional candidate genes associated with fibrogenesis. Collectively, these data established the classification of skin cells and the selective genes expression pattern of cashmere goat.

Table 1. The top 10 genes in per Clusters.

Expression	Cluster 0	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6	Cluster 7
Top1	KRT10 148.51	KRT14 334.64	KRT14 170.19	LOC108634769 232.43	KRT10 232.68	KRT10 322.73	KRT1 197.52	KRT14 194.81
Top2	KRT14 127.88	KRT5 158.27	KRT5 129.94	KRT10 173.85	LOC102177275 179.90	KRT1 197.82	KRT10 155.68	KRT5 112.26
Top3	LOC102177275 108.49	LGALS7 108.19	LOC102177275 110.33	KRT1 128.36	KRT1 132.21	S100A7A 155.10	LOC108634769 116.21	PTMA 95.76
Top4	CST6 101.48	LOC102168701 105.19	RPS8 94.81	RPLP1 93.46	LGALS7 110.19	KRTDAP 99.43	RPLP1 71.73	RPS8 90.80
Top5	KRT5 88.78	TPT1.1 101.40	RPLP1 80.37	LOC102168701 90.56	ACTG1 103.55	SPINK7 97.56	LOC108635081 70.77	LOC1021 87.07
Top6	ACTG1 81.70	RPLP1 98.20	LOC102168701 79.72	RPS8 87.21	CST6 88.81	LGALS7 86.67	LGALS7 66.77	RPLP1 85.31
Top7	KRT1 70.62	RPS8 91.93	TPT1.1 70.67	RPS12 80.54	KRTDAP 84.74	RPLP1 83.42	JUN 61.55	RPS12 67.79
Top8	LGALS7 70.56	S100A2 90.87	RPS12 68.12	S100A7A 67.47	RPS8 80.17	LOC102168701 79.57	KRTDAP 58.47	LGALS7 66.43
Top9	LOC102168701 67.65	RPS12 84.82	LGALS7 66.98	LGALS7 66.95	HSPB1 75.44	SBSN 76.47	RPS8 55.76	RPS19 64.08
Top10	RPLP1 66.06	RPS19 80.19	RPS19 65.51	LOC102169125 64.82	RPLP1 74.39	RPS12 70.96	UBB 55.69	RPS2 60.15

Pseudo-Timeline analysis reveals cell development in cashmere goat skins. To further investigate the classification of skin cell populations and developmental relationship, we perform cell trajectory analysis using the Monocle analysis toolkit¹⁶. To enable a single-cell to differentially expressed genes during development based on their trajectories. Cells were calculated by Monocle 2 with an unsupervised way via maximizing transcription similarity between consecutive cell pairs. Therefore, this method can be used for identify various phases of skin development in cashmere goats. Monocle 2 has a large overlap in each cluster, and the subpopulation of cell classification is distributed in the whole skin, indicating versatility.

To explore which gene regulates the cashmere fiber development progression through per axis, we classified and clustered the gene expression changes with pseudo-time. Pseudo-time results reveal the skin development of cashmere goat is divided into 15 state, every kind of cell is involved in almost all the development process (Fig. 2a, 2b). In support of the draw order in pseudo-time trajectory, our analysis revealed the organizes of genes that performed

differentially expressed in each branch, which closely related to the genes that are famous in participated in cashmere growth and hair development. Interestingly, *KRTAP11-1*, *TCHH*, *KRTDAP* and *S100A8* that were briefly up-regulated and then down-regulated were observed. However, a subset of genes such as *KRT5*, *RPS7*, *RPL3* and *RPL5* were sustained up-regulated over time (Fig. 2c). Further confirming the validity of pseudo-timeline analysis, the top 6 genes such as *KRTAP11-1* and *TCHH* were enriched (Fig. 2d), which GO analysis enriched Intermediate filament and Keratinization are important pathways for cashmere growth, indicating these genes may relate to cashmere formation and development. KEGG pathways of these genes mainly enriched to WNT, TGF beta and PI3K-Akt signaling pathway. These data and analyses revealed the clear developmental stages of goat skins, the differential genes expressions along the time line of bifurcations indicates that different molecular paths guide the development of different populations.

Identification and analysis DPCs andSDPCs in cashmere goat skins. Previous work showed that Dermal papilla cells can induce hair follicle regeneration, determine the size of hair follicle to a certain extent, and play an essential role in hair follicle cycle change process. Dermal papilla cells subtly grasp the continuous growth of mammalian hair, including cashmere. There are 100 high expression genes in DPCs, and the top10 genes exhibited in Fig. 3. *LOC102184693*, *KRT35*, *KRTAP11-1*, *KRTAP3-1* and *MT4* almost only expressed in DPCs. In hair follicle and goat skin, *KRT35* and *KRTAP11-1* are frequently appeared. Moreover, we detected two crucial activators *HOXC8* and *RSPO1* were enriched in DPCs, as reported before¹⁷. In addition, the family of RPS and RPL are significantly expressed in DPCs, such as *RPS8*, *RPS2*, *RPL0* and *RPL32*. *RPS12*, as the key effector of cell competition caused by other RP gene mutations, has the highest expression in DPCs. Among them, *PTGER4* and *ESR1* are more abundant in DPCs consistent with previous studies^{18, 19}. To a better understanding the functioning approaches of differentially expressed genes in DPCs, GO and KEGG enrichment analysis are performed. Finally, 452 pathways were enriched such as keratin filament and hair follicle morphogenesis. By KEGG analysis some pathways related to cashmere growth and development were enriched, including TGF-beta signaling pathway, Alcoholism, PPAR and Notch signaling pathway.

Recently, research in this field has indicated that hair type can be determined by SDPCs. In SDPCs, the top10 up-expression genes exhibited in Fig. 4a, 4b. Of note, *COL1A1*, *COL1A2*, *COL3A1*, *S100A4*, *VIM*, *CRABP2*, *CCDC80*, *ACTG2*, *CCL26* and *CXCL12* were solely abundant in SDPCs. *COL1A2*, *COL3A1* and *CCDC80* were reported to be differentially expressed in different cashmere fibers¹². Furthermore, the expression of *S100A4* was the highest in SDPCs, which is may involve in Yangtze River Delta white goat hair growth²⁰. Also, *NFKB1A* had a highly enriched in SDPCs, which may play an important role in cashmere growth^{12, 21}. Similar, *CRABP1*, a constant marker of DPCs, which has a high expressed in SDPCs, and expresses throughout the whole stages of hair cycling²². In order to further excavate the critical genes regulating cashmere fineness, enrichment was analyzed in SDPCs. A total of 848 GO terms were significantly enriched, as expected, hair follicle morphogenesis, hair follicle development and intermediate filament cytoskeleton were significantly-enriched GO terms. The top 20 differentially expressed GO terms were showed in Fig. 4c. The top 20 pathways by KEGG analysis were significantly enriched, such as PI3K-Akt, TGF beta and WNT signaling pathway (Fig. 4d). *BMP3*, *BMP4*, *BMP7*, *FGF7*, *FGF2*, *COL1A1*, *IGF1*, and *WNT5A* enriched these pathways.

To explore difference genes in development period, we took a closer analyzed of Pseudo-Timeline Analysis of DPCs and SDPCs, respectively. Trajectory analysis provided the development process and five differentiation state in DPCs (Fig 5a, 5b). *CST6*, *KRT10*, *KRTDAP* and *DMKN* decreased first, then rise and then fall at DPCs (Fig 5c, 5d), and yet *KRT35* and *MT4* were momentary upregulated and then downregulated. *SOX4* and *CEBPB* continually upregulated and *KRT14* and *KRT1* downregulated. Pseudo-time analysis demonstrated a total of 5 state in SDPCs development stage (Fig 6a, 6b). *CXCL8* and *CD34*, after a transiently period of stability, it began to downregulated (Fig 6c). Notably, *CXCL4*, *COL1A1*, *COL1A2* and *COL3A1* were continually downregulated and *SRGN*, *NFKB1* and *RGCC* showed late specific expression (Fig 7d). As expected, GO analysis showed that DPCs and SDPCs both significantly-enriched *KRT14*, *CXCL4* and *COL1A1* in the development process, and with the development of pseudo-timeline on the axis, these genes involved in the development were down regulated. *KRT35*, *SRGN* and *NFKB1* showed specific expressions in development later. *CXCL4*, *COL1A1*, *KRT14* and *KRT35* may be the key candidate genes for regulation DPCs and SDPCs.

Weighted Gene Correlation Network Analysis (WGCNA) of cashmere goat skins. In order to further identify the genes related to cashmere growth, we conducted the WGCNA on cashmere goat skins. WGCNA, a relatively new statistical analysis, is usually used to construct networks based on gene correlations and recognize intramodular hub genes²³. The cluster dendrogram consists of 31 co-expression modules, including tan, greenyellow, white, green and other colors (Fig 7a). We also found that compared with others, the blue module has a strong co-expression relationship by building a topological overlap matrix (TOM) diagram (Fig. 7b). The darker the color, the higher the topological overlap.

As shown in Fig. 7c, all genes in 13 type cells were selected to produce the module trait relationship. Interestingly, before there is almost no similarity between each module, suggesting that most modules had certain promoting effect on each cluster. The significantly enrichment modules in secondary hair follicles dermal papilla cells is blue, in dermal papilla cells is green, and in stromal cell is gray60, and the number of genes in per module was 2629, 1839, and 256, respectively. The blue module of SDPCs cluster related to cashmere growth has the deepest color, suggesting that genes in this module may have a positive effect on cashmere fineness. We identified hub gene *COL1A1*, *COL1A2*, *CCL26*, *CCDC80*, *HOXA13*, *CXCL8*, *KRT24*, and *BMP3* has significant connectivity, which may associate with cashmere development.

Enrichment analysis was used to explore the biological functions of genes in these modules, to further determine the relationship between these genes and cashmere growth. The GO analysis results of the core genes in blue module were displayed in Fig. 7d. It was found that these genes are suggested to cytoplasm, integral component of the membrane.

The detailed network information of core genes for 4 selected modules including blue, brown, green and gray60 modules was showed in Fig 8. In the gene networks, some hub genes interact with many other genes, which indicates that they are more likely to be necessary. *COL1A1*, *CCDC80* and *HOXA13* are associated with many genes in blue module. *CXCL8*, *NFKB1E* and *CCL24* are actively in green module. *KRT3*, *KRT4* and *KLK12* are positively linked to other genes in gray60 module. *KRT19*, *KRT38*, *NFIB* and *LHX2* are positive genes in brown module. *COL1A1*, with a significant connectivity in SDPCs, may serve as a target for cashmere formation. IF staining with *COL1A1* showed that *COL1A1* is expressed in SDPCs, which is as predicted by scRNA-seq and WGCNA (Fig. 9).

Analysis and verification of genes difference in SDPCs of CT-LCG and FT-LCG. A total of 16601 genes were found in SDPCs of two samples (Fig. 10a), there are 15 representative DEGs between CT-LCG and FT-LCG exhibited in Fig. 10b. *TCHH*, *S100A8* and *IGF2* were highly expressed in CT-LCG, nevertheless, *CD74*, *CCL27*, *COL1A1* and *CXCL8* were highly expressed in FT-LCG. In order to further excavate the critical genes regulating cashmere fineness, enrichment was analyzed in SDPCs. There are 48 and 45 significant pathways in CT-LCG and FT-LCG. CT-LCG are mainly enriched to Jak-STAT signaling pathway, TGF-beta signaling pathway and PI3K-Akt signaling pathway (Fig. 10c), and FT-LCG also enriched in TGF-beta signaling pathway (Fig. 10d). *COL1A1* enriched to PI3K-Akt signaling pathway and *CXCL8* enriched to NF-kappa B signaling pathway, which had been reported may be related to cashmere.

We identified *ACTA2* (α -SMA) as a marker gene of SDP cells by immunofluorescence, α -SMA protein was almost exclusively expressed on secondary hair follicles, as shown in Fig. 11. To further determine the relationship between these genes and cashmere fineness, we validated *COL1A1* and *CXCL8* in SDPCs by immunofluorescence assay. We have made immunofluorescence test on the skin of three coarse and three fine Liaoning Cashmere Goats. The differential expression of *COL1A1* in coarse and fine type skin indicates that the number of SHFs in fine skin is more distributed, arranged tightly and with smaller cashmere diameter, while the number of SHFs in coarse skin is less distributed, arranged sparsely and with larger cashmere diameter (Fig. 12). Wang have shown that *COL1A1* has difference in CT-LCG and FT-LCG²⁴. In the results of immunofluorescence, the nucleus is blue and the target protein is green. PHF is the primary hair follicle, SHF is the secondary hair follicle, which is the place where cashmere grows. SeG is sebaceous glands. They form the hair follicle cluster. The SDPCs exist in the secondary hair follicles. *CXCL8* is the differential expression gene of CT-LCG and FT-LCG screened by us. The result shows that the expression level of *CXCL8* (IL-8) protein in CT-LCG is higher than that in FT-LCG (Fig. 13). From the results, we can see that the results of immunofluorescence are consistent with the results of single-cell sequencing.

The OD value of protein standard was measured at the wavelength of 568 nm, and the standard curve was established. The regression equation and variance coefficient were $y = 0.6965x + 0.0141$ and $R^2 = 0.997$. The results showed that the expression level of *ACTA2* (α -SMA) and *CXCL8* in FT-LCG was lower than that in CT-LCG, as shown in Fig. 14.

Discussion

Cashmere goats mainly produce cashmere fiber, which plays an indispensable role in textile industry. Cashmere fineness, the characteristics of natural quality, is a very important evaluation index in cashmere production. The study on cashmere diameter is the core issue of cashmere industry development. However, the research on the fineness of cashmere is limited, and there is almost no research on cell level. The development of single-cell RNA-seq not only enriches the research methods of identifying cell types in cashmere goat skins, but also provides more candidate genes for hair formation regulation. Previous studies have used single cell sequencing technology to reveal the molecular signatures of human, mouse and other animals^{25, 26, 27, 28}.

Here, we used single-cell sequencing to recognize 13 clusters of cells in Liaoning Cashmere Goat skins, including Dermal papilla cells, Secondary hair follicle dermal papilla cells and Stromal cells. We detected highly expressed genes in every cell type, and the development process of goat skin was studied by Pseudo Time Analysis. Differential gene expression profiles of 13 clusters provide a unique opportunity to identify positive markers to study the function of rare and insufficient cashmere goat skin. Then, we identified 31 modules in 13 types of cells through WGCNA, and the blue module was the most significant one in the SDP related to cashmere growth. Finally, we identified 16601 genes and fifteen typical differentially expressed genes were screened in SDPCs between CT-LCG and FT-LCG, and *COL1A1* and *CXCL8* were verified. Our current study provides the first step to fill in the knowledge gaps about the characteristics of skin cell types in cashmere goats.

The Pseudo time analysis of cashmere goat skin divided into 15 stages and 7 nodes. With the development of time axis, *KRTAP11-1*, *KRTDAP* and *TCHH* play a role in early development, and *KRT5*, *PPS7* and *RPL5* play a role in development later. The results showed that most of these genes were active in the later stage of skin development. GO analysis enriched to Intermediate filament and Keratinization, and KEGG pathway analysis mainly enriched to WNT, TGF beta and PI3K-Akt signaling pathway, indicating these genes may relate to cashmere formation and development. The WGCNA results have enriched 31 modules in total, and we have carried out network analysis on 4 modules. *COL1A1*, *CCDC80*, *HOXA13*, *NFKBIE*, *CCL24*, *KRT3*, *KRT4*, *KRT19*, *KRT38*, *NFIB* and *LHX2* are the key genes in co-expression analysis module. *COL1A1* is an important gene of the result of Pseudo Time Analysis and WGCNA in SDPCs of cashmere goat, which may be related to cashmere formation.

Dermal papilla cells act as a relay station to transmit the effects of hormones and other molecules produced locally or systematically on hair growth²⁹, which also can instruct the activity of follicular keratinocytes, to reshape hair follicle and produce new hair shaft. Also, previous studies have demonstrated that the induction characteristics of DPCs play a key role in morphogenesis, development and hair formation of hair follicles and regulates the hair growth cycle after birth^{22, 30}. It is well known that some characteristic molecules are considered as classic signs to evaluate the induction characteristics of dermal papilla cells, such as *HSPC016*, *Wnt10b*, *BMP6* and *Wnt5a*^{30, 31, 32, 33}. However, their roles in the regulation of DPCs secretion and their molecular mechanisms are still unclear. In our study, we observed that *KRT35*, *KRTAP11-1* and *KRTAP3-1* have unique expressed in DPCs. *SOX2*, *WNT10b*, *HOXC8* and *RSPO1* also have high expressed in DPCs. *SOX2* as a specific marker gene in dermal papilla cells and its restricted expression in guard²². *Wnt10b* can accelerate the proliferation of DPCs, and also has a potent ability to maintain *VERSICAN* expression in DPCs and sustained hair follicle³². Additionally, Ma defined 25 core signatures of cashmere goat DPCs, such as *HOXC8* and *RSPO1*¹⁷.

Recently, a considerable number of genes have been identified that might be involved in reconstruction and development of cashmere goat secondary hair follicle. Wang reported some genes, such as *HOXC13*, *SOX9*, *JUNB*, *LHX2*, and *GATA3*, involved in hair follicle differentiation through selective expression in embryonic day 120³⁴. In recent years, studies about cashmere goat secondary hair follicles dermal papilla cells has increased^{35, 36, 37, 38}, however, few information was available associated with the development and cashmere fineness of Liaoning Cashmere Goat SDPCs. In our investigations, we demonstrated that some genes exhibited significantly different expression in secondary hair follicle dermal papilla cells, such as *COL1A1*, *COL3A1* and

CCDC80. *COL1A1* (Collagen type I $\alpha 1$ is a major component of collagen type I. The study has showed that *COL1A1* can regulate the growth of Alpaca villi³⁹. Collagen 1 alpha 1 (*COL1A1*) is suggested to participate in the progression of fibrotic diseases and wound healing. In single cell sequencing of mouse skin, dermal and keratinocyte populations were identified using *COL1A1* for dermal populations⁴⁰. *COL1A1* can promote the proliferation, migration and invasion of rectal adenocarcinoma cells, but inhibit their apoptosis. *COL1A1*, as a hub gene, affects the proliferation, invasion, migration and apoptosis of rectal adenocarcinoma cells by weighted correlation network analysis. The expression level of *COL1A1* is related to primary human pulmonary fibroblasts (pHPFs). *COL1A1* variants are associated with the risk of acute musculoskeletal soft tissue injuries⁴¹. *S100A4* had the highest enriched in SDPCs, which had been discovered in the human hair follicle dermal papilla cells³⁸.

At present, our analysis revealed *ACTA2* as marker gene of SDPCs that could be used to genetic and breeding development tool. *ACTA2* encodes alpha smooth muscle actin (alpha-SMA). Bergeron et al modeled two human *ACTA2* mutants P.(Asn117Thr) and P.(Arg118Gln) in yeast actin, a mutant highly similar to smooth muscle actin⁴². *ACTA2* knockout promotes progressive cavity expansion, apoptosis and phenotypic regulation of Vascular Smooth Muscle Cells (VSMCs) in mice⁴³. Studies of *ACTA2* gene mutations have shown that mutation carriers exhibit a variety of vascular lesions, including early-onset coronary artery disease (CAD), early-onset ischemic stroke (including moyamoya disease), and familial thoracic aortic aneurysms and dissecting aneurysms (TAADs)⁴⁴. Studying the mutations of alpha-SMA and *ACTA2* genes is of great significance for understanding the pathogenesis of vascular lesions and determining the relevant treatment strategies for vascular lesions⁴⁵.

In addition, we identified 15 DEGs in two kinds of fiber fineness Liaoning Cashmere Goat skins, and *CXCL8* has a significant difference in SDPCs of CT-LCG and FT-LCG. *CXCL8*, a member of CXC chemokine family, is an inflammatory cytokine produced by neutrophils, macrophages, endothelial cells and cancer cells. *CXCL8*, a member of the CXC chemokine family, is an inflammatory cytokine produced by neutrophils, macrophages, endothelial cells and cancer cells. *CXCL8* is an important factor in angiogenesis, which is thought to play an important role in the process of tumorigenesis. *CXCL8* could induce angiogenesis⁴⁶. *CXCL8* is a proinflammatory chemokine that mediates its signaling through extracellular binding to two G-protein-coupled receptors, C-X-C chemokine receptor type 1 (*CXCR1*) and C-X-C chemokine receptor type 2 (*CXCR2*)⁴⁷. It overexpressed in pancreatic cancer (PC) cell lines⁴⁸. *CXCL8* promotes the proliferation and metastasis of a colorectal cancer (CRC) cell line. *CXCL8* may inhibit apoptosis of CRC cells⁴⁹. *CXCL8* is an important cytokine. Mast cells can induce the expression of *CXCL8* in CSCC cells and participate in tumor growth, invasion and neovascularization⁵⁰. With respect to the SDPCs signature genes, we found that *COL1A1* has unique expressed and *CXCL8* has a obvious difference.

In conclusion, we identified 13 kinds of cells and the differentially expressed genes in the skins of cashmere goats. The skin development of cashmere goat can be divided into 15 stages through the analysis of pseudo time. According to the network constructed by WGCNA, 31 modules were determined and 4 modules were selected for detailed analysis, among which the genes in blue module was the most significant in SDPCs. The results of scRNA-seq and immunofluorescence demonstrated that *COL1A1* may acted to the regulation skin development and *CXCL8* may regulate cashmere fineness in Liaoning Cashmere Goats. Furthermore, our study provides valuable resources for the identification of cell map of formerly unknown cashmere goats, which in turn will help to study the special contribution of cell to cashmere fiber.

Potential implications

Cashmere fineness is one of the important factors determining cashmere quality, however, our understanding about the cells that make up the cashmere fineness is limited. We identified the key genes regulating cashmere formation in cashmere goat skin by 10xGenomics single-cell sequencing. These genes may be involved in regulating the fineness of cashmere in goat secondary hair follicle dermal papilla cells, our research will provide new insights into the mechanism of cashmere growth and cashmere fineness regulation by cells.

Methods

Ethics. The methods were performed in accordance with relevant guidelines and regulations and approved by the Laboratory Animal Management Committee of Shenyang Agricultural University, animal welfare number is 201806019.

Tissue dissociation and preparation of single-cell suspensions. The skin tissue of cashmere is taken from two healthy Liaoning cashmere goats from the Liaoning Animal Husbandry Research Institute. The cashmere fineness of the two goat is 17.19 μ m and 14.3 μ m. Place a sterile RNase-free culture dish containing an appropriate amount of calcium-free and magnesium-free 1 \times PBS on ice, the tissue was transferred into the culture dish and cut it into 0.5 mm² pieces, the tissues were washed with 1 \times PBS, and remove as many non-purpose tissues as possible such as blood stains and fatty layers. Tissue s were dissociated into single cells in dissociation solution (0.35% collagenase IV, 2 mg/ml papain, 120 Units/ml DNase I) in 37 $^{\circ}$ C water bath with shaking for 20 min at 100 rpm. Digestion was terminated with 1 \times PBS containing 10% fetal bovine serum (FBS $\text{\textcircled{V}}$), then pipetting 5-10 times with a Pasteur pipette. The resulting cell suspension was filtered by passing through 70-30 μ m stacked cell strainer and centrifuged at 300g for 5 min at 4 $^{\circ}$ C. The cell pellet was resuspended in 100 μ l 1 \times PBS (0.04% BSA) and added with 1 ml 1 \times red blood cell lysis buffer (MACS 130-094-183, 10 \times) and incubated at room temperature or on ice for 2-10 min to lyse remaining red blood cells. After incubation, the suspension was centrifuged at 300g for 5 min at room temperature. The suspension was resuspended in 100 μ l Dead Cell Removal MicroBeads (MACS 130-090-101) and remove dead cells using Miltenyi $\text{\textcircled{R}}$ Dead Cell Removal Kit (MACS 130-090-101). Then the suspension was resuspended in 1 \times PBS (0.04% BSA) and centrifuged at 300 g for 3 min at 4 $^{\circ}$ C (repeat twice). The cell pellet was resuspended in 50 μ l of 1 \times PBS (0.04% BSA). The overall cell viability was confirmed by trypan blue exclusion, which needed to be above 85%, single cell suspensions were counted using a Countess II Automated Cell Counter and concentration adjusted to 700-1200 cells/ μ l.

Chromium 10x Genomics library and sequencing. Single-cell suspensions were loaded to 10x Chromium to capture single cell according to the manufacturer's instructions of 10X Genomics Chromium Single-Cell 3' kit (V3). The following cDNA amplification and library construction steps were performed according to

the standard protocol. Libraries were sequenced on an Illumina NovaSeq 6000 sequencing system (paired-end multiplexing run, 150bp) by LC-Bio Technology co.ltd., (Hangzhou, China) at a minimum depth of 20,000 reads per cell.

Bioinformatics analysis. Using 10 x Genomics official analysis software Cell Ranger (<https://support.10xgenomics.com/single-cell-geneexpression/software/overview/welcome>) to filter, compare, quantify and identify the recovered cells, and finally get the gene expression matrix of each cell. Seurat (<https://satijalab.org/seurat/>) was used for further cell filtration, standardization, classification of cell subsets, differential expression gene analysis of each subgroup and Marker gene screening. We performed cell filtration based on the number of genes expressed by cells (using the gene expression number 500 as the threshold). The filtering of low-quality cells uses Seurat data analysis R package. its functions include quality control, filtering, data standardization, PCA analysis, tSNE differential gene analysis, etc. at the same time, it has the function of visualizing the analysis results⁵¹. Seurat analyzed the differentially expressed genes in different cell populations by bimod⁵² likelihood ratio statistical test to screen up-regulated genes in different cell populations.

To visualize the data, we further reduced the dimensionality of all 9771 cells using Seurat and used t-SNE to project the cells into 2D space. The steps includes: 1. Using the LogNormalize method of the "Normalization" function of the Seurat software to calculate the expression value of genes; 2. PCA (Principal component analysis) analysis was performed using the normalized expression value, Within all the PCs, the top 10 PCs were used to do clustering and t-SNE analysis³. To find clusters, selecting weighted Shared Nearest Neighbor (SNN) graph-based clustering method. Marker genes for each cluster were identified with the bimod with default parameters via the find all markers function in Seurat. This selects marker genes which are expressed in more than 10% of the cells in a cluster and average log (Fold Change) of greater than 0.25. For cell trajectory analysis, we used Monocle analysis toolkit⁵³, Monocle 2 analysis was used to order cells in pseudo time. We use the default function to estimate the size factor and dispersion. Applying DDRTree for dimension reduction. To visualize all pseudo chronous genes that may regulate cashmere development, we cluster the regulation of genes according to time changes.

Gene Ontology (GO <http://www.geneontology.org>) and KEGG (Kyoto Encyclopedia of Genes and Genomes) are the functions of Kyoto Gene and Genome Encyclopedia (www.genome.jp/kegg) to analyze the function of differential genes. The results are displayed using ggplot set analysis scatterplot.

WGCNA analysis. The co-expression network was performed using "WGCNA" R package for the filtered genes⁵⁴. We use pairwise Pearson coefficient to find the co-expression adjacency matrix of all genes in the weighting. The topological overlapping measure (TOM) matrix is used to estimate its connectivity in the network⁵⁵. The co-expression network analysis of hub gene in modules was established by Cytoscape 3.5.1 software. Each node represents a gene associated with a different number of genes.

Immunofluorescence. Skin tissue used for this test was derived from six Liaoning Cashmere Goats, including three fine type goats and three coarse type goats. The goat number of the three fine goats were 78413, 78623, 76201, and their cashmere fineness is 14.32 μ m, 14.69 μ m and 14.77 μ m, respectively. The goat number of three coarse goats is 79031, 77401, 79105, and their cashmere fineness is 17.23 μ m, 17.63 μ m and 17.91 μ m, respectively. The skin tissues were fixed with 4% paraformaldehyde for 24 hours, dehydrated with gradient alcohol, treated with xylene transparently and embedded in paraffin. Slides were sliced by Leica pathological slicer (Leica RM 2016 rotary slicer, Germany). The slides were put into a 40 °C water bath pot for spreading. The anti stripping glass piece is inserted into the water surface obliquely to remove the slice, so that the slice is attached to the appropriate position of the slides. Bake the slice in a 60 °C oven (Wuhan Junjie jk-6 biological tissue spreading and baking machine) for 3 hours. The paraffin sections were successively put into xylene (20min) - xylene (20min) - xylene (20min) - anhydrous ethanol (5min) - anhydrous ethanol (5min) - 95% alcohol (5min) - 90% alcohol (5min) - 80% alcohol (5min) - 70% alcohol (5min), and then soaked in distilled water for 5min. Microwave was used for antigen repair. The dewaxed and hydrated tissue sections are placed on the high temperature resistant plastic section frame in the beaker (or repair box), and a proper amount of repair solution (0.01M citric acid buffer, pH6.0) is added in the beaker. The liquid surface should be soaked in the slides to a certain height. The microwave oven can first use high-grade heating to make the liquid boil. When heating to boiling, adjust it to the middle level, and start timing, the repair time is 15 minutes. Take out the beaker from the microwave oven, put it into cold water to cool down, take out the glass slide when the repair solution drops to room temperature, wash it with PBS (pH7.4) for 3 times, each time for 3 minutes. Dry the slides with absorbent paper, draw a circle around the tissues with the brush of the immune group, drip diluted normal goat serum, and seal it at room temperature for 30 minutes to reduce non-specific staining. Shake off the excess liquid, do not wash it, then drop the diluted first antibody, after adding the first antibody, incubate it in a 4°C wet box overnight (15h). The sections were washed by BST for 3 times, 3 minutes each time. After the sections were dried by absorbent paper, the diluted fluorescent secondary antibody was added. The slides were incubated in a wet box at 37 °C for 1 hour. The slides were washed by PBST for 4 times, 3 minutes each time. The sections were washed by BST for 3 times, 3 minutes each time. After the sections were dried by absorbent paper, the diluted fluorescent secondary antibody was added. The sections were incubated in a wet box at 37 °C for 1 hour. The sections were washed by PBST for 4 times, 3 minutes each time. Primary antibody: collagen I (Wuhan doctoral Bioengineering Co., Ltd.), ACTA2 (Abcam), IL-8 (Abcam), dilution ratio is 1:100. Secondary antibody: fluorescence (Cy3) labeled Sheep anti rabbit IgG (Abcam), fluorescence (Cy3) labeling of Goat anti mouse IgG, dilution ratio is 1:100.

Western Blot. The proteins in each group were extracted using Radio Immunoprecipitation Assay (RIPA) lysis buffer added with protease inhibitors. Then, quantification of protein was performed with bicinchoninic acid (BCA) Protein Assay Kit. The extracted protein supernatant was mixed with 5 \times sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis (PAGE) loading buffer (the volume ratio was 4:1) and put into boiling water for 10 minutes. Prepare electrophoresis gel as shown in Table 2. Fix the prepared glue on the electrophoresis tank, and pour the electrophoresis solution into the storage tank. The prepared protein sample and marker were added into the sample hole with a micro sampler, and the total protein content of each sample was 40 μ g. After adding the sample, first constant pressure 80V electrophoresis to the Bromphenol blue indicator at the junction between the concentrated glue and the separation glue, and change to constant pressure 120V to Bromphenol blue to the bottom of gel, this process is about 1.5h. Take out the gel, cut the target strip according to Marker, rinse with distilled water, cut the same size polyvinylidene fluoride (PVDF) film and filter paper with the PAGE gel, immerse the PVDF membrane with methanol for a few seconds, and immerse it in the electromigration buffer with the filter paper. According to the black plate - fiber mat - filter paper - gel - PVDF membrane - filter paper - fiber mat - white plate in good order, after clamping plate into the rotating film instrument, black plate side contrast

black negative pole. Fill the membrane transfer tank with electric fluid to start membrane transfer. The PVDF membrane was soaked in tris buffered saline tween (TBST) (blocking solution) containing 5% skimmed milk powder and sealed in a shaker at room temperature for 2 hours. The PVDF membrane was immersed in the primary antibody incubation solution and incubated overnight at 4 °C. The antibody dilution concentration is as shown in Table 3. The PVDF membrane was fully washed by TBST for 5 times, 5min / time. Put up to 3 films in a dish, and pay attention to whether the film is attached to the dish wall or whether the films overlap during membrane washing. TBST was used to dilute the corresponding HRP labeled second antibody — 1:50000, and the PVDF membrane was immersed in the second antibody incubation solution and incubated in a shaker at room temperature for 2 h. The PVDF membrane was fully washed by TBST for 5 times, 5min / time. Mix the reinforcement solution and stable peroxidase solution of electrochemiluminescence (ECL) reagent in the proportion of 1:1, drop the working solution onto PVDF membrane, react for several minutes, after the fluorescence band is obvious, use filter paper to absorb the excess substrate solution, cover with fresh-keeping film, press the X-ray film, then successively put the developing solution, fixing solution and develop the film. Dry the film, scan the film, and analyze the gray value of the film with IPP. β -actin was used as a loading control.

Table 2 Configuration system of electrophoretic gel

Gel concentration	Component	Volume /ml
5% Concentrated gum		6
	H ₂ O	4.1
	30% Acrylamide	1.0
	1.0Mtris-HCl pH6.8	0.75
	10%SDS	0.06
	APS	0.06
	TEMED	0.006
12% Separating gum		20
	H ₂ O	6.6
	30% Acrylamide	8.0
	1.5Mtris-HCl pH8.8	5.0
	10%SDS	0.2
	APS	0.2
	TEMED	0.008

Table 3 Primary anti dilution ratio

Primary antibody name	Dilution ratio
β -actin	1:1000
Collagen I	1:1000
ACTA2	1:1000
CXCL8	1:1000

Declarations

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

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Author's Contributions

Zeying Wang, Wenlin Bai, Rina Wu and Yanxu Zhu design the project; Yuanyuan Zheng, Yanru Wang and Taiyu Hui carry out the experiment; Xingtang Dou, Xinghui Zhang and Dan Guo provide resources; Jiaming Sun, Weidong Cai, Xinjiang Zhang, Ming Gu, Yuting Qin perform and isolate sample collection; Yuanyuan Zheng, Yanru Wang, Taiyu Hui, Yanan Xu, Yu Zhang, Suping Guo, Chang Yue, Zhixian Bai perform the bioinformatic analysis; He Tian, Wei Wang, Yuyan Cong, Man Bai, Yixing Fan perform the soft; All authors reviewed the manuscript.

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Figures

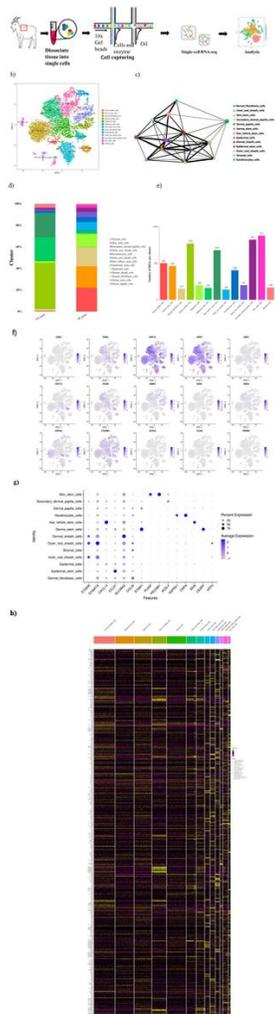


Figure 1

Identification of cashmere goat skin cell types. a) Schematic representation of cashmere goat skin tissue preparation ufor single-cell transcriptome analysis. b) t-SNE clustering of cells based on genes expression levels. Each color represents a class of cells. c) Interaction between cells. d) Graph showing percent of each clusters in two samples. e) Number of DEGs in each cluster. f) t-SNE plots of marker genes expression value in per cluster. g) Highly expressed genes in every cell type. h) Heatmap illustrating the top ten genes in each cluster.

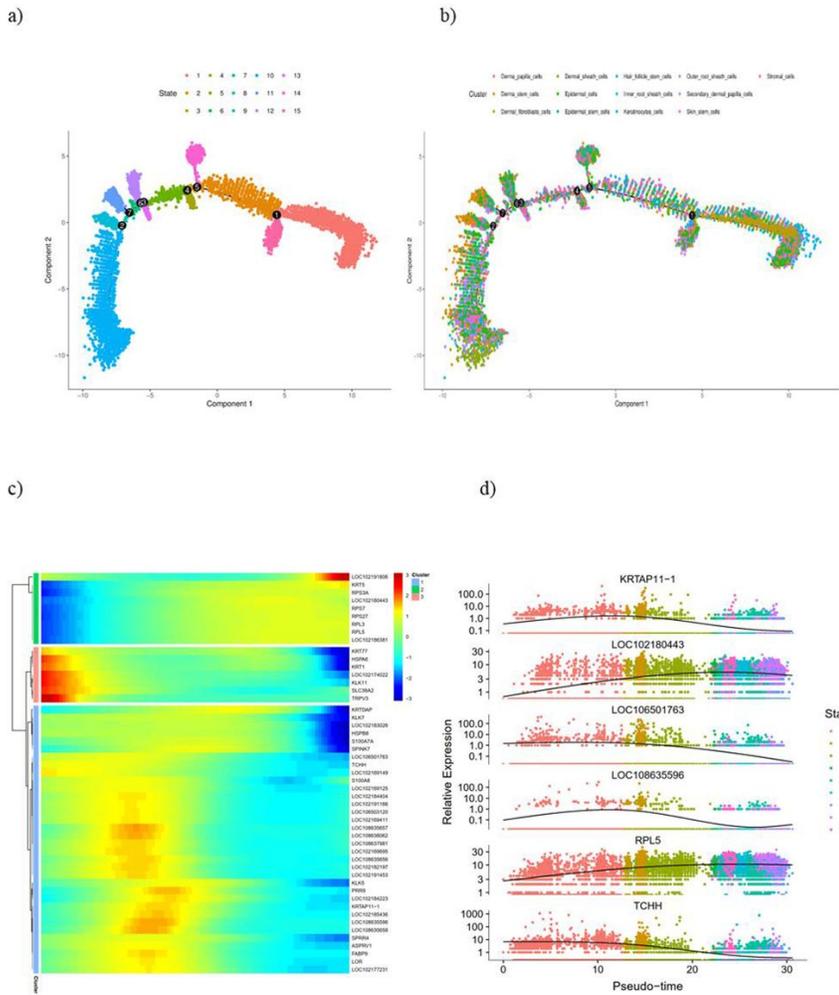


Figure 2

. Pseudo-Timeline Analysis on goat skin cells. a) Monocle2 pseudo time trajectory of goat skin cells. b) Monocle2 pseudo time trajectory colored according to 13 goat skin cells identity. c) Clustering of genes represent differential expression patterns across goat skin cell populations. d) Dynamic expression values of top six different genes are plotted on Pseudo-Time. X-axis represents pseudo time, and Y-axis represents gene expression levels.

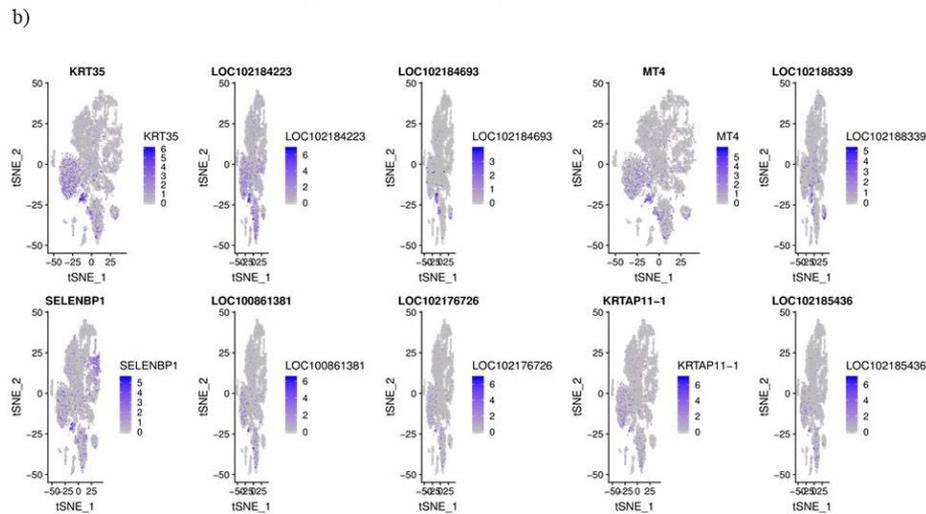
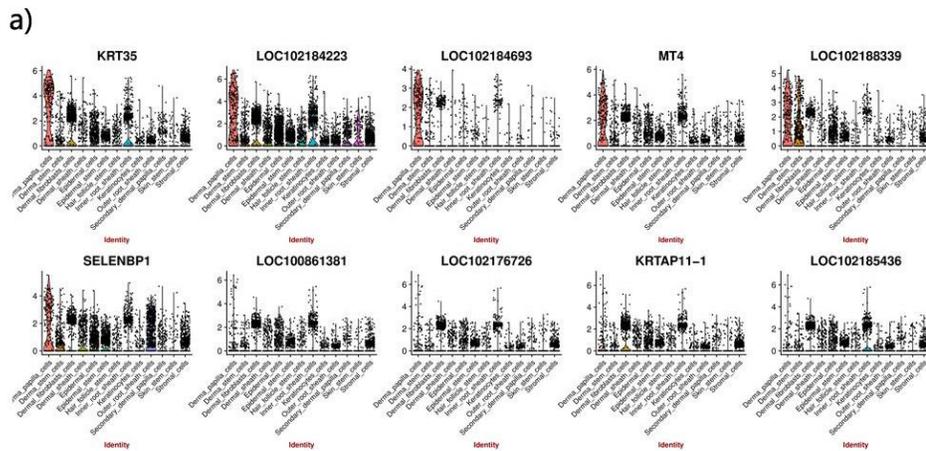


Figure 3

Genes expression profiling of DPCs. a) Violin plots for expression of top ten genes. b) Relative expression levels of top ten genes on the t-SNE plots.

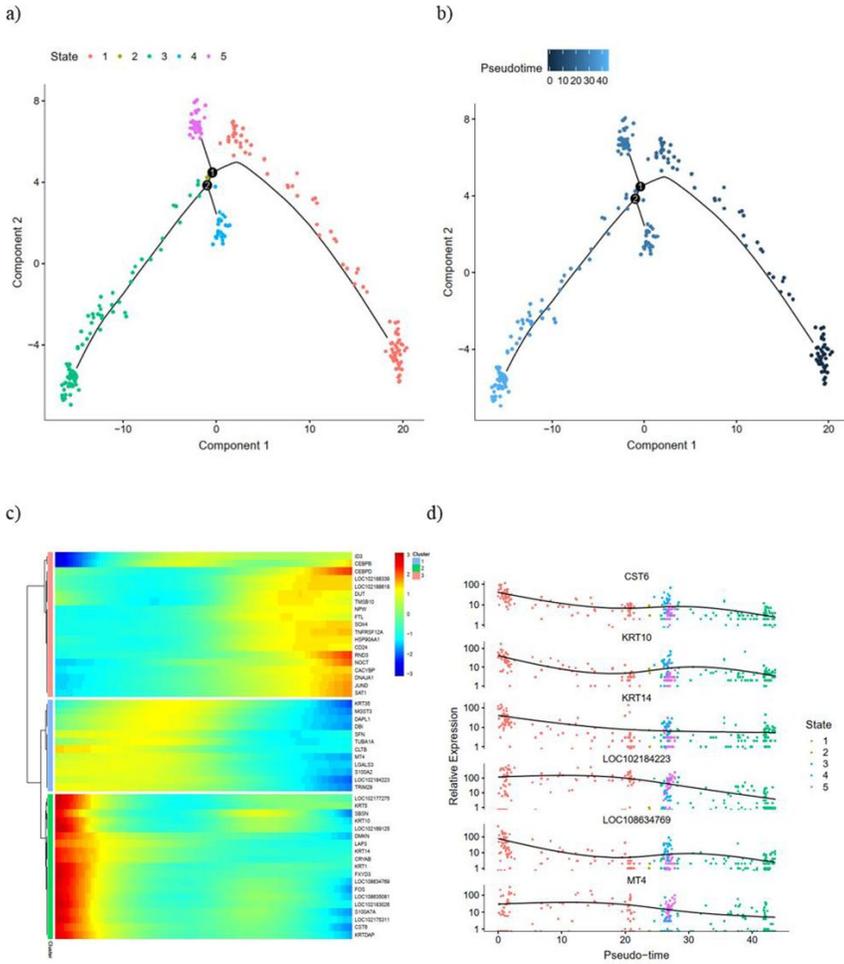


Figure 5

Pseudo-Timeline Analysis in DPCs of goat skins. a) Monocle2 pseudo time trajectory of goats DPCs. b) Pseudo time is displayed in a dark to light blue gradient and indicates the start of pseudo time. c) Clustering of top 50 genes exhibiting in DPCs. d) Expression levels of 6 responsive genes in DPCs. X-axis represents pseudo-time, Y-axis represents genes expression levels.

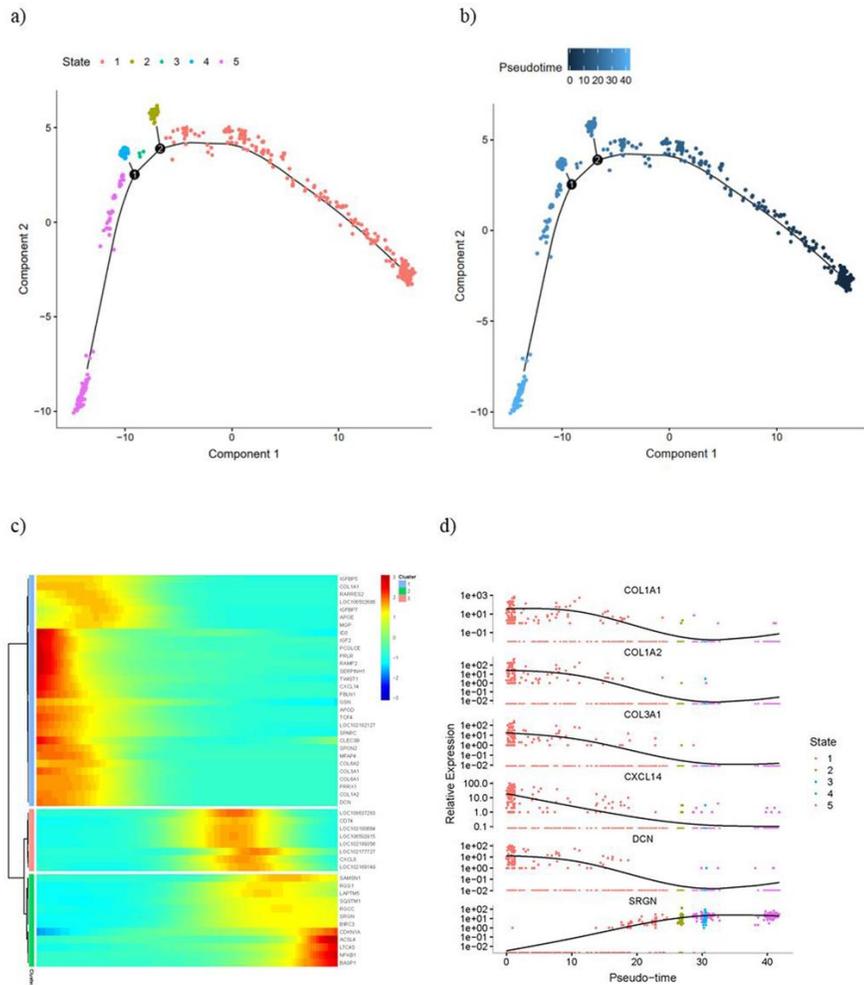


Figure 6

Pseudo-Timeline Analysis in SDPCs of Goat Skin. a) Monocle2 pseudo time trajectory of goat DPCs. b) Pseudo time is displayed in a dark to light blue gradient and indicates the start of pseudo time. c) Clustering of top 50 genes exhibiting in SDPCs. d) Expression levels of 6 responsive genes in DPCs. X-axis represents pseudo-time, Y-axis represents genes expression levels.

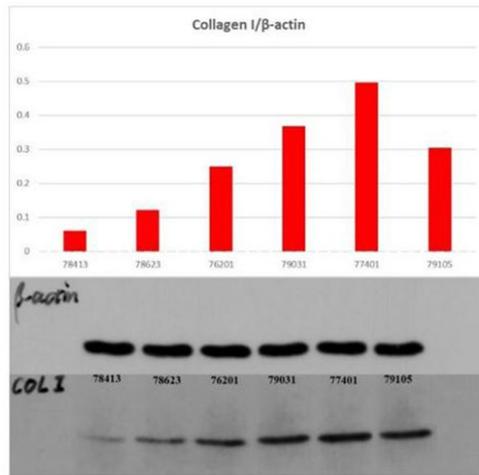
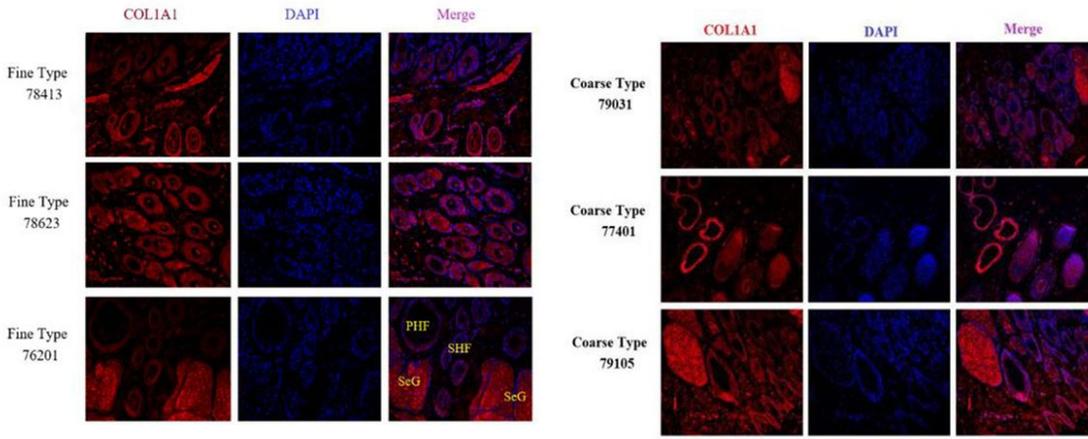


Figure 9

Immunofluorescence results of COL1A1 in FT-LCG and CT-LCG skin tissues (200x).

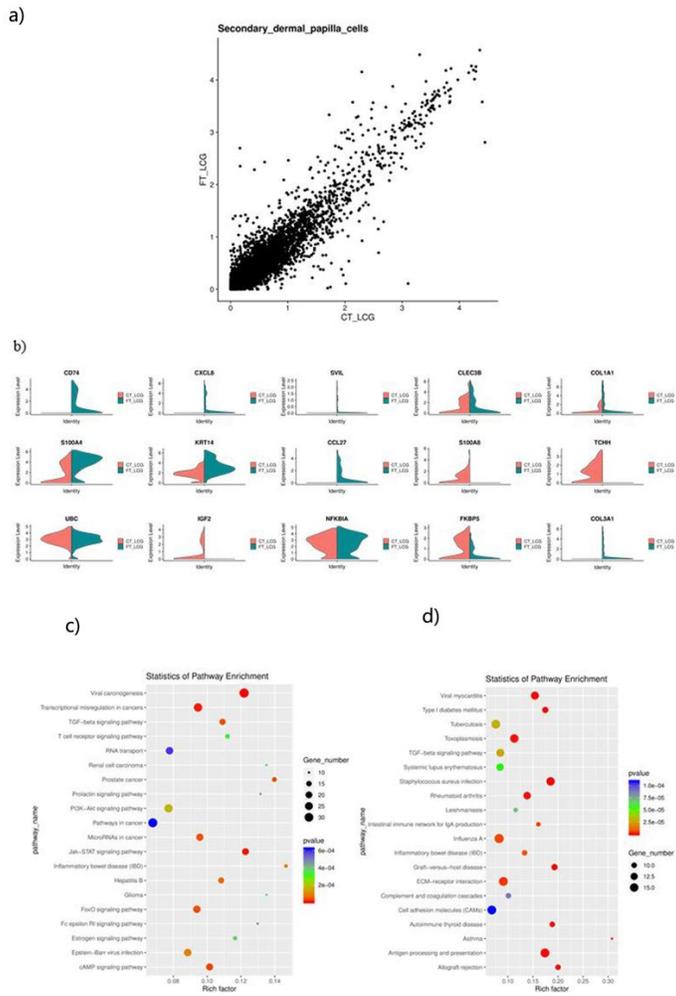


Figure 10

Analysis of differentially expressed genes between CT-LCG and FT-LCG SDPCs. a) Volcanic graph of differentially expressed genes in SDPCs. b) Top 20 up-expressed genes in SDPCs. Red: CT-LCG; Green: FT-LCG. c) KEGG analysis in CT-LCG. d) KEGG analysis in FT-LCG.

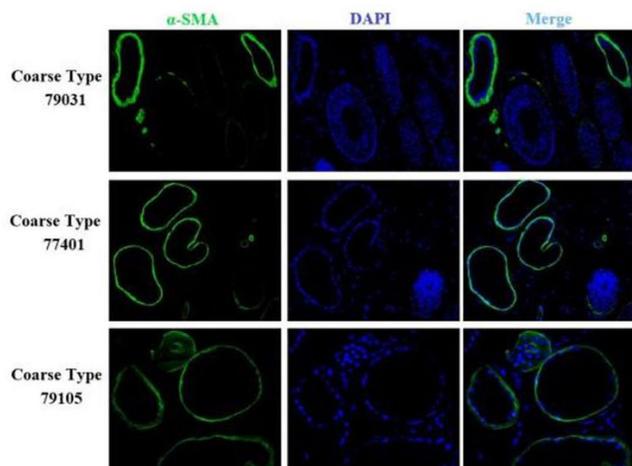
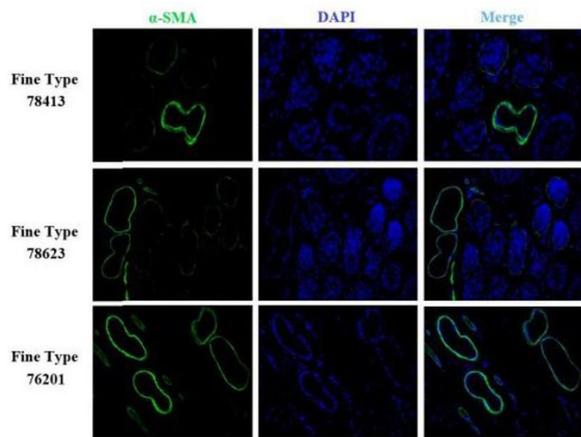


Figure 11

Immunofluorescence results of ACTA2 (α -SMA) in FT-LCG and CT-LCG skin tissues (200x).

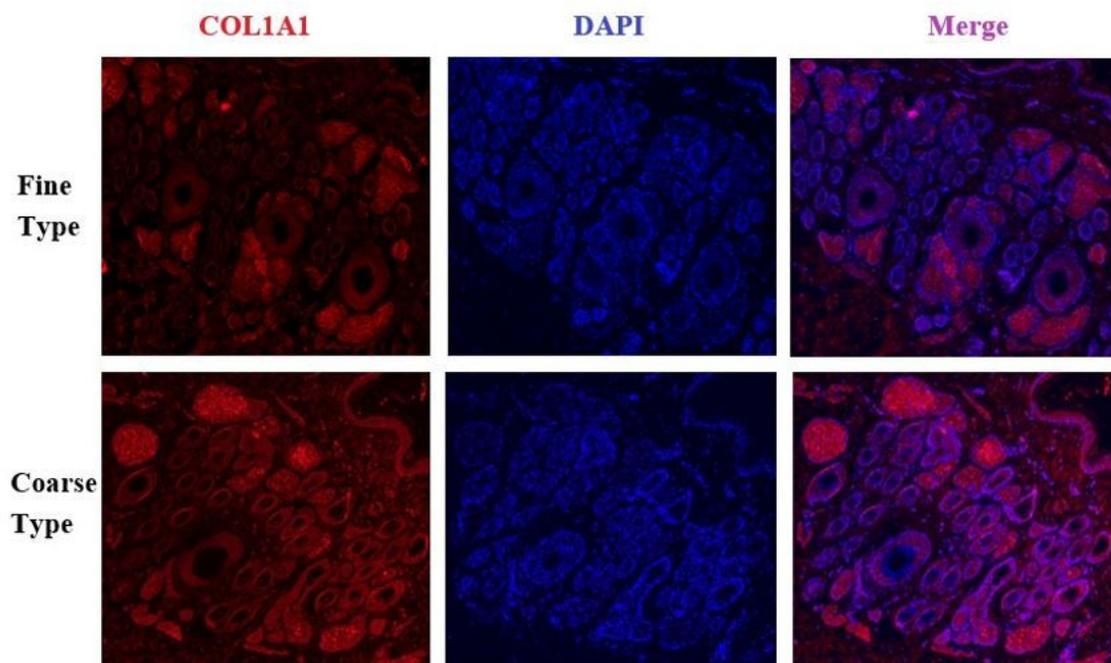


Figure 12

Immunofluorescence results of COL1A1 in FT-LCG and CT-LCG skin tissues (40x).

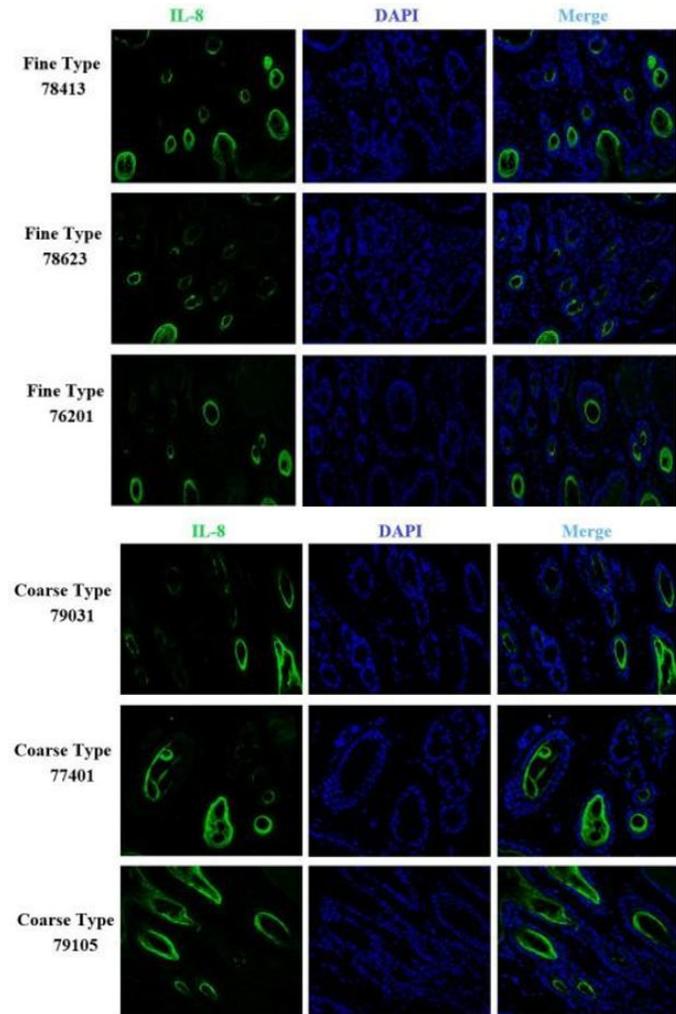


Figure 13

Immunofluorescence results of CXCL8 in FT-LCG and CT-LCG skin tissues (200x).

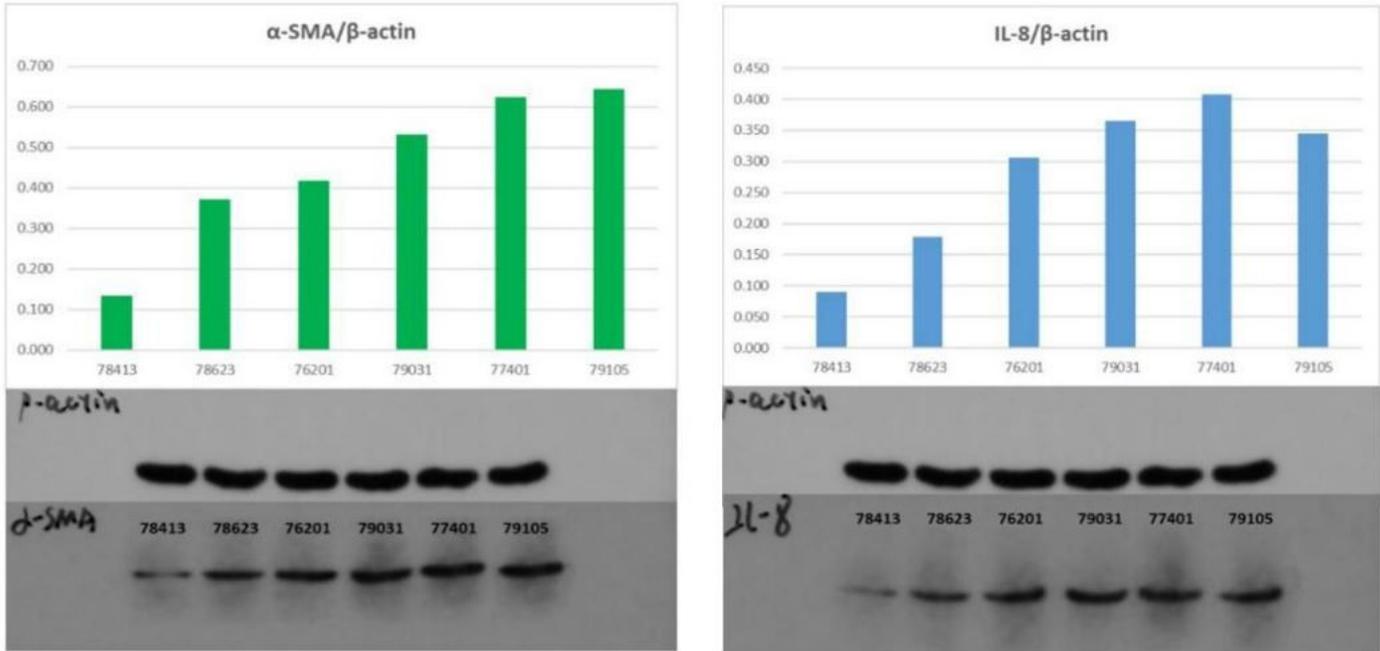


Figure 14

The relative expression of ACTA2 (α-SMA) and CXCL8 (IL-8) at CT-LCG and FT-LCG at protein level.