Single-cell sequencing reveals the intermediate cell state and function of dermal papilla cells in the hair follicle cycle of cashmere goats

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Abstract

Background:

Human hair loss and regeneration has stimulated interest in the natural hair cycle worldwide; however, such research is difficult because the periodicity of human or mouse hair is not visually obvious. Dermal papilla cells (DP cells) play an important role in the development of hair follicles, but knowledge of the differentiation and mechanisms of DP stem cells during transition through the hair follicle cycle are still limited, although some studies have reported that DP cells may have an intermediate cell state during differentiation, the classification and function of specific cell states are not clear.

Results:

Here, we used cashmere goats, that have obvious periodicity of hair follicles, as model animals and, based on unbiased single cell RNA sequencing, we identified and isolated DP cell data. Pseudotime ordering analysis was used to successfully construct a DP cell lineage differentiation trajectory and revealed the sequential activation of key genes, signaling pathways, and functions involved in cell fate decisions. At the same time, we analyzed the mechanisms of different cell fates and revealed the function of four different intermediate cells: Intermediate cells 10 showed important functions in the growth of cashmere and maintenance of cashmere attachment to the skin; intermediate cells 1 revealed important functions in the process of apoptosis and cashmere shedding of secondary hair follicles; intermediate cells 0 initiated new follicular cycles and completed the migration of hair follicles and the occurrence of cashmere; and intermediate cells 15 are suggested to be DP progenitor cells.

Conclusions:

In development and apoptosis, inner bulge cells not only earlier than outer bulge cells, but occurred faster and was more thorough,this helps a deeper understanding of the role of bulge cells. Pseudogenes play another important role in function which promoted the competitive endogenous RNA (ceRNA) hybridization of pseudogenes.In different hair follicle cycles, DP cells will differentiate into different intermediate state cells and perform different functions, and the marker genes of the cells also changed. Intermediate cells 10 showed important functions in the growth of cashmere and maintenance of cashmere attachment to the skin; intermediate cells 1 revealed important functions in the process of apoptosis and cashmere shedding of secondary hair follicles; intermediate cells 0 initiated new follicular cycles and completed the migration of hair follicles and the occurrence of cashmere; and intermediate cells 15 are DP progenitor cells, this conclusion provides an unprecedented deeper understanding of the function of DP cells.

Introduction

Hair follicle development has been widely studied [1–4] and results show that they can have obvious periodicity [1, 5, 6]. In particular, the development of secondary hair follicles in cashmere goats can be divided into three stages: anagen, catagen, and telogen [7, 8]. During the embryonic stage, the unspecified epidermis receives signals from the mesenchyme (“first dermal signal”) and subsequently forms layers of thickening epidermis known as placodes; these are the earliest morphological characteristics that mark the initiation of hair follicle morphogenesis [9, 10]. Plaque sends out feedback signals and epithelial signals to promote the aggregation and coagulation of mesenchymal cells to form the initial structures of dermal papilla. During anagen, a dermal signal causes a series of gene and signal pathway changes, leading to hair follicle morphology [11]. Following the “second skin signal” [12], a series of signal transmissions [13] finally promote epithelial proliferation and down growth to form the hair follicle structure and to generate secondary hairs.

Dermal papilla (DP) cells are mesenchymal cells in the hair follicle, which regulate the development of the hair follicle and the growth of secondary hairs [14]. Many reports have revealed that DP cells are pluripotent stem cells [15, 16]. Previous studies have shown that the number of DP cells determines the number of hair follicles and the density of secondary hairs, and have revealed the heterogeneity of DP cells in different types of hair follicles [17, 18]. The hair of cashmere goats is divided into primary hair (guard hair) and secondary hair (ground hair or cashmere) [19, 20]. The cashmere quality of cashmere goats has a significant relationship with the secondary hair follicles [21], but the specific regulatory mechanism has not been fully explained. Therefore, the mechanism of action of DP cells on cashmere growth needs to be elucidated.

There are many complex cell types in the hair follicle structure. At present, DP cells, bulge cells, Hari germ (Hg) cells and matrix cells have been studied and described [20, 22–24]. Mok et al. show that during early hair follicle development, the molecular markers of specific cell groups may change during cell state change [25], some of which are intermediate cells; however, these intermediate cells have not been clarified. DP cells have many types of cell interference and different intermediate cell states, so research into their mechanisms has proved to be difficult.

Using single cell RNA(scRNA) seq to solve these problems, a recent study describes the status of intermediate cells formed during hair follicle development, which helps to analyze the fate of DP cells and determine the unrecognized intermediate cell status during development [26]. In the current study, we generated 4000 single cell scripts of cashmere goat dorsal skin from telogen and anagen. By using t-distribution random neighbor embedding(tSNE), we identified six major cell populations and isolated DP cells. Based on Monocle pseudotime ordering analysis, we successfully constructed the DP cell lineage differentiation trajectory and revealed the sequential activation of key genes, signaling pathways, and functions involved during cell fate decisions. Along with this, we analyzed the mechanism of different cell fates, which provided a molecular landscape for the action of DP cells during the initiation of the hair follicle cycle. Taken together, our data provided new insights into DP cell fate decisions during the hair follicle cycle, revealed the function of intermediate cells differentiated by DP cells, and delineated molecular information regarding the underappreciated DP cytodifferentiation stages.

Results

In order to decipher the transcriptome regulatory network and cell fate decisions of DP cells during the hair follicle cycle, we isolated and dissociated dorsal skin tissue of cashmere goats from telogen (HFT) and anagen (HFA), into single cells and performed droplet-based single cell RNA seq (Fig. 1A). After deleting the low-quality cells, we detected 17 045 genes from 1000 cells in telogen and 19 371 genes from 3000 cells in anagen (Supplementary Fig. 1A). Overlapping information of cell distribution and gene expression were obtained (Supplementary Fig. 1B, C). In order to analyze the heterogeneity of cells during the hair follicle cycle, we performed tSNE clustering of all the single cells (Fig. 1B), we found that only a small fraction of cells overlapped between telogen and anagen (Fig. 1B, left), in addition, tSNE clustering analysis revealed 16 cell clusters according to their gene expression profiles (Fig. 1B, right). Subsequently, we evaluated a series of well-recognized marker genes which we clustered, revealing seven major cells: *Lum* was highly expressed in dermal cells [27], *Pecam1* was highly expressed in endothelial cells [28], *Gata3* was highly expressed in matrix cells [29], *Fcer1g* was highly expressed in immune cells [30], *Sox9* was highly expressed in bulge cells [31], and *Plp1* was highly expressed in melanocyte cells [32] (Supplementary Fig. 2A); the genes that were highly expressed in DP cells were *Lef1, Msx1, Pcna*, and *Hoxc13* (Fig. 1C)[22, 33–35]. These analyses together completed the true characterization of different cell populations (Supplementary Fig. 2B, C, Supplementary Table 1). In order to isolate the DP cell population, we performed further expression analysis of DP marker genes (Fig. 1D, Supplementary Table 2), and found that *LEF1, MSX1, and HOXC13* were highly expressed in cell clusters 0, 1, 10, and 15, and *Pcna* was highly expressed in cell clusters 0, 1, 3, 4, 5, 6, 7, 10, 12, 14, and 15 (Supplementary Fig. 3A). *Msx1, Lef1, Hoxc13* revealed the true characterization of DP cells; using the *Lef1* gene as a marker gene, we created interactive plots to further characterize DP cells (Supplementary Fig. 3B). We finally determined that cell clusters 0, 1, 10, and 15 were DP intermediate cell populations (Fig. 1E). In addition, we also revealed the expression pattern of all cell marker genes (Supplementary Fig. 3C). In summary, we successfully identified the major cell types in the telogen and anagen skin of cashmere goats and identified cell-specific characterization genes. These characteristic genes enabled true cellular heterogeneity identification using single-cell RNA sequencing.

DP Intermediate Cell Separation And Cell Fate Determination

We initially performed pseudotime ordering of all cells based on the Monocle algorithm (Fig. 2A). After analysis, we observed that the DP cell population was mainly concentrated in state2, 3, and 4 of branch point 1, and state5 of branch point 2 (Supplementary Fig. 4A). We then extracted all DP cells and performed re-pseudotime ordering analysis (Fig. 2B). DP cells had four branch points in the pseudotime ordering of hair follicle processes. Cell cluster 0 was mainly concentrated at branch points 1 and 3; cell cluster 1 was mainly concentrated at branch points 2 and 4; cell cluster 10 was concentrated at pre-branch of the branch point 4; and cell cluster 15 was concentrated at the pre-branch of branch 4 and branch point 3 (Supplementary Fig. 4B). In addition, based on pseudotime ordering, we analyzed the

expression levels of four marker genes in DP cells (Fig. 2C). *Pcna* was highly expressed in state 1, 2, 8, and 9 of cell cluster 0; *Msx1* and *Lef1* were highly expressed in all states of cell clusters 0 and 1; and *Hoxc13* was highly expressed in state 5 and 6 of cell clusters 1 and 10. Finally, we performed all gene expression analyses along each branch (Fig. 2D) and combined these with tissue observations (Fig. 2E) to reveal DP cell mechanisms and intermediate cell transition nodes. First, we visualized the gene expression profile (Fig. 2D), divided the highly expressed genes into four genesets, and then enriched the GO functions and the KEGG signaling pathways (Supplementary Fig. 5A, Supplementary Tables 3 and 4). The following KEGG signaling pathways were enriched: FOXO-mediated transcription, Wnt signaling pathway, NOTCH signaling pathway, MAPK signaling pathway, BMP signaling pathway and complexes such as LARC complex, and PCNA-MSH2-MSH6 complex. GO functions included: epithelial cell differentiation, epidermis development, skin development, keratinocyte differentiation, epidermal cell differentiation, and cornification. We revealed the main signaling pathways and the functions of DP cells. Epithelial cell differentiation and epidermal development revealed the source of DP cells, and immunofluorescence showed the development of embryonic hair follicles (Fig. 2F). Keratinocyte differentiation revealed the regulation of DP cells during cashmere growth. It is worth noting that we enriched the rhythmic process and melanogenesis. We speculated that hair follicle development is rhythmic and that melanocytes respond to daylight intensity as an inducer of hair follicle activation. In addition, we studied the relationship between genesets and found that there is an important interaction between genesets (Supplementary Fig. 5B), which promote the formation of DP cells, and the growth of cashmere. At the same time, we visualized the GO functions and the KEGG signal pathways (Supplementary Fig. 5C), further revealing the GO and KEGG distribution of DP cells, and visualizing the interaction network between proteins (Supplementary Fig. 6), depicting the molecular landscape of DP cell protein interactions.

The transition of intermediate cells from cell cluster 10 to cell cluster 1: A foreshadowing of hair follicle apoptosis

In order to reveal the changes in DP cell fate and cell status, we first analyzed branch point 4, named it cell fate 1 (Fig. 3C, top), and performed gene expression analysis along branch point 4 (Fig. 3A, Supplementary Table 3). For pre-branch (geneset2), we enriched the compound catabolic process, but did not enrich the functions or signaling pathways associated with hair follicle development. For geneset1, we enriched keratinization, keratinocyte differentiation, epidermal cell differentiation, epidermis development, skin development, aging, and hair cycle (Fig. 3B, top). These functions are more like the process of maintaining guard hair growth and adhering to the skin, although they were enriched with epithelial cell differentiation, this was not sufficient to demonstrate the initiation of hair follicle development. Therefore, we initially speculated that this process was a continuation of the maintenance of the old hair follicle cycle; that is, the maintenance of hair follicle state during the telogen period, but before guard hair fall out (Fig. 3E). It is worth noting that we enriched the negative regulation of cell proliferation, and cytokine-mediated signaling pathway in geneset2, which further proved our inference. In order to further reveal this process, we extracted genes associated with GO functions (Fig. 3B, bottom), and found that most of these genes were keratin gene families and keratin-related protein gene families.

We then visualized these genes (Fig. 3D) and found that their expression showed a decreasing trend according to pseudo-time ordering, which revealed the cause of the gradual decrease of hair follicles in observed hair follicle tissue. It is worth noting that *KRT10* was included in these genes (Fig. 3D, geneset1). In previous studies, *KRT10* was found to be the marker gene for outer bulge cells, and *Krt6a* the marker gene for inner bulge cells (Fig. 4, geneset1). *Krt6a* began to decline at an earlier stage, and it can be speculated that during root sheath cell apoptosis, the inner root sheath cells undergo apoptosis first. Subsequently, we enriched all KEGG signaling pathways at branch point 4: TP53 regulates metabolic genes, aging, regulation of epidermis development, transcriptional regulation by TP53, interleukin-10 signaling, Naba secreted factors, cytokine-mediated signaling pathway, and negative regulation of cell proliferation (Supplementary Fig. 7A). In particular, the negative regulation of cell proliferation further revealed the process of hair follicle maintenance towards the end of the telogen period. Finally, we re-pseudotime ordered all the cells in branch 4 (Fig. 3C, bottom), and found two branch points, which were composed of intermediate cells 1 and 10; intermediate cells 10 evolved toward intermediate cells 1, indicating that the period was still in telogen, but had just started to evolve to anagen. We then compared the pseudotime ordering analysis of gene expression between DP and branch point 4 cells (Supplementary Fig. 7B). As we thought, these genes were highly expressed in all cells of cell fate 1, but were only highly expressed in branch point 4 of pseudotime ordering in all DP cells, the emergence of intermediate cell state 1 marks the end of the process of keeping cashmere from falling out. In general, intermediate cells 10 played an important regulatory role in the process of hair follicle maintenance, and our conclusions support the evidence that the inner bulge cells apoptosis earlier than the outer bulge cells.

Intermediate cell 1 played an important role in hair follicle apoptosis and intermediate cell cluster 0 was the key cell for hair follicle development

In order to reveal the cell fate of branch 2, we performed gene expression analysis on cell fate 2 (branch point 2; Fig. 4A) First, focusing on the KEGG signaling pathways (Supplementary Table 3), we enriched FOXO-mediated transcription, p53 signaling pathway, PID REG GR pathway, and maintenance of location in cell (Supplementary Fig. 8A), which indicated apoptotic processes in hair follicles. For geneset1, we enriched keratinization, keratinocyte differentiation, epidermal cell differentiation, epidermis development, skin development, cornification, epithelial cell differentiation, formation of the cornified envelope, aging, maintenance of location in cell, and hair cycle (Fig. 4B, top). This result is consistent with cell fate 1, indicating that the geneset1 process of cell fate was a continuation of cell fate 1; we extracted the major genes (Fig. 4B, bottom) and visualized them (Fig. 4D, geneset1). Compared with cell fate 1, cell fate 2 had 21 more genes. The expression trend of these genes was the same as that in cell fate 1; gene expression was gradually reduced according to pseudotime ordering, which further illustrated that the geneset1 process was an apoptotic process of hair follicles. It is worth noting that this process not only included the keratin gene family and the keratin-associated protein gene family, we also extracted many regulatory genes such as *Csta, Dap, Cnn3, Penk*, etc. These genes have similar expression patterns to those of the keratin gene family. Similarly, we visualized the *Krt6a* gene which is the marker gene of inner bulge cells, and the expression level showed a decreasing trend. Compared with the outer bulge cell marker gene

Krt10, the decreasing trend was more obvious, except for State5; it was almost no longer expressed in other states, which indicated that the apoptosis of inner bulge cells was earlier, faster, and more thorough than that of outer bulge cells. Unfortunately, we had not enriched any GO functions or KEGG signaling pathways associated with hair follicle development in geneset2 and geneset3, but it is worth mentioning that a large number of pseudogenes were found in geneset2: *Loc102169182, Loc102189548, Loc102182562, Loc102171808, Loc108634465, Loc102184991, Loc102179869, Loc108637496, Loc102175427*, and *Loc102180996*; these pseudogenes accounted for 84% of the total genes of geneset2, and they were all ribosome-related pseudogenes. These pseudogenes attracted our interest, but we did not investigated whether they had a role at present. We speculated that in the process of apoptosis, the regulation of sRNA or genes can promote normal genes to be transcribed into pseudogenes and then inhibit their expression, thus cooperating with the process of hair follicle apoptosis. In addition, we randomly extracted several genes in geneset2 and geneset3 for visualization (Fig. 4D, geneset2 and geneset3), and found that the expression patterns of these genes were completely different from those of geneset1. The expression level was increased according to pseudotime ordering; this attracted our attention, so we extracted all the cells in cell fate 2 and performed re-pseudotime ordering (Fig. 4C). Unexpectedly, in cell fate 1, the intermediate cell cluster 10 began to evolve to 1, but they had almost disappeared in cell fate 2. Meanwhile cell cluster 1 occupied a major proportion, and had evolved to cell states 0 and 15 at the end of pseudotime ordering. Thus, we further compared the expression of these genes in the pseudotime ordering of DP cells and cell fate 2 cells (Supplementary Fig. 8B). In geneset2 and geneset3, genes were highly expressed in cell state changes (Fig. 4C, bottom), combined with signaling pathways changes; we speculated that DP cells began to receive upstream signals at this location, and that new hair follicle development occurred during the transition from telogen to anagen. This result is consistent with previous studies, that the secondary hair follicles of cashmere goats entered anagen at the end of March; our tissue observations (Fig. 2E, Mar. and Fig. 4E) also confirmed these results. In cell fate 1, we also observed cell cluster 15, and it doesn't seem to be directly related to hair follicle development. From this, we speculated that intermediate cell 1 played an important role in hair follicle apoptosis and intermediate cell 0 was the inducing factor in hair follicle development during transition from telogen to anagen and is a key cell in hair follicle development.

Cell fate 3 reveals the development of hair follicles in anagen

In order to verify the conclusion in cell fate 2, we analyzed cell fate 3. First we revealed the gene expression pattern in branch point 3 (Fig. 5A) and found that geneset1 was still dominant in the process. There were 307 genes involved in this process, accounting for 45% of the total genes of branch point 3 (Supplementary Fig. 9B). We enriched GO functions with tissue cell development, skin development, epidermal cell differentiation, keratinocyte differentiation, and cornification and keratinization (Fig. 5B, top and Supplementary Fig. 9A and Table 3), indicating that the process of old cycle cashmere growth and attachment to the skin continued. Furthermore, we enriched with formation of the cornified envelope, epidermis development, hair follicle development, myelination, gliogenesis, and hair cycle and epithelial cell differentiation; these results indicated that the new hair follicle cycle had begun and the hair follicles were beginning to develop. It is worth mentioning that we had previously enriched melanosome transport.

In another study we have shown that melanocyte-receiving signals are the triggers for the initiation of the hair follicle cycle. Here, DP cells also promoted the development of the hair follicle cycle by receiving melanocyte signals. In addition, we analyzed the expression of genes in geneset1 (Fig. 5E, geneset1) and the protein interaction network (Supplementary Fig. 9F, left). The expression of these genes decreased along with pseudotime ordering, further indicating that these genes were associated with cashmere growth, the previous cashmere fall off with the decline of gene expression.. We also enriched the KEGG signaling pathways: apoptotic signaling pathway, Rab protein signal transduction, PID DELTA NP63 pathway, PID P73 pathway, PID BETA CATENIN NUC pathway, PID P53 downstream pathway, ERAD pathway, and PID MTOR 4 pathway (Supplementary Fig. 9A, geneset1). Among them, PID BETA CATENIN NUC pathway and apoptotic signaling pathway further illustrated that geneset1 was the process of hair follicle apoptosis and cashmere shedding in the last cycle. For geneset2, we enriched GO functions: DNA replication, cell cycle, synthesis of DNA, rhythmic process, connective tissue development, gland development, osteoblast differentiation, stem cell differentiation, glial cell development, tissue morphogenesis, and tissue migration; these functions marked the development of new cycle hair follicles. In particular, osteoblast differentiation and stem cell differentiation played a crucial role in the initiation of the hair follicle cycle. It is worth noting that we also enriched the rhythmic process, from which we speculated that the hair follicle cycle transition is indeed rhythmic, in addition to being controlled by genes and hormones. At the same time, we enriched the KEGG signaling pathways and major complexes of geneset2 (Supplementary Fig. 9A, geneset2). It is worth mentioning that we enriched the TNFR2 non-canonical NF-kB pathway, Wnt signaling pathway, signaling by NOTCH, and MAPK6/MAPK4 signaling. These signaling pathways further revealed the hair follicle development process, and the protein interaction network of geneset2 also supported this conclusion (Supplementary Fig. 9F, middle). We extracted 258 genes from geneset2 (Supplementary Fig. 9B) and visualized the important genes. (Fig. 5E, middle). It was found that the expression levels of these genes increased significantly along with pseudotime ordering. For geneset3, we enriched eukaryotic translation, translation, cytoplasmic translation, signaling by ROBO receptors, ncRNA processing, and TNF-alpha/NF-kappa B signaling, which provide the energy for hair follicle development, in which the highly expressed genes included *Eif3e, Rpl* gene family, and the *Sox* gene family. In order to reveal the function of cells during hair follicle cycle transition, we performed re-pseudotime ordering of cells in cell fate 3 (Fig. 5C, bottom), and found that the main cells involved in the process were cell cluster 0 and cell cluster 1 which involved crosstalk between the apoptosis of old ground hairs and the development of new hair follicles; this further explained the function of intermediate cell 1 and intermediate cell 0. The comparison of the pseudotime ordering of DP cells and cell fate 3 cells also revealed the effect of gene expression in cell cluster 0 and cell cluster 1 on old cashmere apoptosis and new hair follicle development (Supplementary Fig. 9C). In general, cell fate 3 revealed the apoptosis of old cashmere and the development of new hair follicles (Fig. 5F), especially in the new cycle, where genes, proteins, and environmental interactions contributed to the development of hair follicles.

DP cell ultimate fate: hair follicle development and new cashmere growth

Finally, we focused on cell fate 4 and performed gene expression analysis along branch 1 (Fig. 6A); we then enriched the GO functions and KEGG signaling pathways for these gene expression profiles (Fig. 6B and Supplementary Fig. 10A, B). We visualized the relationships between these functions and signaling pathways (Fig. 6D), and examined the interaction of genes and proteins (Fig. 6E, upper-left and Supplementary Fig. 10C, D). These results revealed that cell fate 4 was the golden age of hair follicle development and was the stage of transition from hair follicle development to cashmere growth. For geneset1, we enriched the KEGG signaling pathways with cell cycle, PID FOXM1 pathway, regeneration, signaling by NOTCH3, and FoxO signaling pathway (Supplementary Fig. 10A). The genes in these signaling pathways fulfilled the following functions of the hair follicle cycle: gland development, developmental growth, epithelial cell differentiation, connective tissue development, tissue regeneration, cilium assembly, vasculature development, epithelial cell migration, and tissue migration (Fig. 6B, top and Supplementary Fig. 10A), These functions indicated the intricacy of the hair follicle structure, especially connective tissue development, tissue morphogenesis, cilium assembly, and tissue migration. Furthermore, they revealed the complexity of hair follicle development, describing the development of other tissues, including glands, connective tissue, blood vessels, etc. These tissues achieved functions such as the fixation of hair follicles, the supply of nutrients, and the function of cashmere in regulating body temperature, moisture, and metabolism. During this period, the hair follicle migrates down the dermis and connects to the capillaries to provide nutrients for cashmere growth. In addition, we visualized some important genes in geneset1 (Fig. 6E, upper right) and protein interaction networks (Supplementary Fig. 10E, geneset1 PPI). The results showed that *Pcna, Plk1, Tk1*, and *Top2a* were mainly expressed in state1 of cell cluster 0, this result revealed that the function of geneset1 was mainly achieved in cell cluster 0. We enriched the GO functions and KEGG signal pathways of geneset2, including positive regulation of cell migration, cell morphogenesis involved in differentiation, homotypic cell-cell adhesion, maintenance of cell number, cell-cell communication, regulation of cell morphogenesis involved in differentiation, stem cell population maintenance, stem cell division, and osteoblast differentiation (Supplementary Fig. 10A, geneset2). These functions illustrate the three directions of DP cells: Firstly stem cell morphology changes, cells differentiate and divide to maintain stem cell populations and thus control cashmere types. Secondly, the same type of cells adhere to each other to form cell masses and migrate upwards to be surrounded by matrix cells. Finally, DP cells communicate with other cell types to complete hair follicle development and cashmere growth. After that, we paid attention to the signaling pathway in geneset2, and the following were enriched: PI3K-Akt signaling pathway, PID AR TF pathway, signaling by receptor tyrosine kinases, response to cAMP, rhythmic process, PID BETA CATENIN NUC pathway, signaling by PDGF, and PID P38 MK2 pathway. These signaling pathways are important for hair follicle development, we also enriched the GO functions with extracellular structure organization, collagen formation, collagen fibril organization, and elastic fiber formation, which complement the hair follicle structure. In particular, appendage morphogenesis further illustrated the generation and growth of cashmere at this stage. Visualizing genes in geneset2 (Fig. 6E, bottom left) and protein interaction networks (Supplementary Fig. 10E, geneset2 PPI), we found that the expression patterns of these genes were between geneset1 and geneset3, indicating that geneset2 was the process of transition from hair follicle development to cashmere growth. So we further analyzed geneset3 and found that the following

were enriched: ribosome, cytoplasmic, eukaryotic translation initiation, protein localization to endoplasmic reticulum, cytoplasmic translation, epidermis development, keratinization, keratinocyte differentiation, epidermal cell differentiation, skin development, cornification, and formation of the cornified envelope (Supplementary Fig. 10A) These GO function results were similar to cell fate 1, but the gene and protein interactions expressed in geneset3 (Supplementary Fig. 10E, geneset3 PPI) were different from cell fate 1. This result revealed the production of new cashmere and was consistent with the results from June to September in the organizational observations (Fig. 2E, June–Sept. and Fig. 6F). It is worth noting that the old cashmere falls out before the new cashmere are produced. In the re-pseudotime ordering of cell fate 4, cell cluster 1 was almost non-existent, which further predicted the function of cell cluster 0 in hair follicle development. In general, cell fate 4 illustrated the process of hair follicle development, in addition, we re-pseudotime ordered cell fate 1 cells (Fig. 6C, bottom), and revealed the process of intermediate cell 0 ending its mission and apoptosis.

In total, we have summarized and revealed the function of four different intermediate cells: Intermediate cells 10 showed important functions in the growth of cashmere and maintenance of cashmere attachment to the skin; intermediate cell 1 showed important functions in the processes of apoptosis and cashmere shedding; intermediate cell 0 initiated a new follicular cycle, completed the migration of hair follicles and the growth of cashmere. It is worth noting that cell cluster 15 existed at all stages of hair follicle development, and the number of cells was relatively stable; we therefore speculated that cell cluster 15 was a DP progenitor cell. From this, we summarized the cell fate of DP cells in the hair follicle cycle (Fig. 6G). Total DP cells can be divided into three fates in the hair follicle cycle: Telogen cell fate, anagen cell fate, and catagen cell fate. Telogen cell fate mainly describes the function of DP cells on cashmere maintenance and attachment to the epidermis; anagen cell fate reveales the function of DP cells on hair follicle development and cashmere growth; catagen cell fate describes the process by which DP cells return to their original state after completing their function (inferred only by cell state, no in-depth study has been performed).

Finally, Western blot analysis was used to detect the expression of LEF1 protein (Fig. 6H left), which indirectly verified the change rule of the number of DP cells. The results showed that the expression of LEF1 increased significantly in the transition from telogen to anagen, and continued to increase in the transition stage from anagen to catagen (Fig. 6H right), indicating that the number of DP cells increased during hair follicle development. This agreed with our single cell transcriptome sequencing results. But because the number of hair follicles increased at the same time, we could not determine the number of DP cells in each hair follicle. In general, Western blot results revealed the quantitative changes of DP cells during the development of hair follicles.

Discussion

Crosstalk of asynchronous development of primary and secondary hair follicles, similar marker genes for different types of hair follicle cells, and intermediate cell states involved in hair follicle morphogenesis are the main obstacles to our understanding of the development of secondary hair follicles. However, in mice,

seminal works have revealed the molecular signatures of different hair follicle cells and their roles during hair follicle development. Although, the conclusions raised by different groups vary, there are still two main questions to be answered: first, the role of molecular mechanisms of DP cells in the development of secondary hair follicles; second, the inentification of DP cells in intermediate cells of hair follicle development.

Single-cell transcriptomics describe many complex biological processes in a detailed and comprehensive way [36], because it offers strong analytical ability regarding cell heterogeneity, which is conducive to the revelation of functions of different cell types in complex tissues. Recently, by using scRNA seq, seminal works have delineated an underappreciated intermediate pre-DP fate transition stage occurring prior to DC formation [26]. Here, we used the same technique to perform scRNA seq on hair follicles at two time points and isolate DP cells to provide a comprehensive understanding of genes, proteins, and signaling pathways. To determine DP cell fate, we observed four intermediate cell states. Interestingly, in DP cells, the gene expression of the four intermediate cells were significantly different, and the marker genes also changed. The marker genes *Top2a, Stmn1, Cdk1, Ptma*, and *Kif11* were highly expressed in intermediate cells 0; *Znf618, Notch1, Rpl14, Krt35, Tle4, Hoxc13*, and *Lef1* were highly expressed in intermediate cells 1; *Smpd3, Krt39, Wnt31, Foxn1, Krtap11-15*, and *Sun23* were highly expressed in intermediate cells 10; and *Krt23, Foxq1, Stra6, Prph, Th1, Fgf22*, and *Cnfn* were highly expressed in intermediate cells 15. These together emphasized the difference in the expression of intermediate cell genes and cell transition status.

In order to reveal the function of DP intermediate cells, we performed pseudotime ordering analysis of these cells and analyzed different intermediate cells. For the GO function of cell fate 1, we enriched keratinization, keratinocyte differentiation, epidermal cell differentiation, epidermis development, skin development, aging, and hair cycle. These functions revealed the processes of growth or maintenance of cashmere [37]. For the signaling pathways we enriched TP53 regulates metabolic genes, aging, regulation of epidermis development, transcriptional regulation by TP53, interleukin-10 signaling, NABA secreted factors, cytokine-mediated signaling pathway, and negative regulation of cell proliferation; in particular, the upstream gene of the P53 signaling pathway, *Tp53*, predicts apoptosis of hair follicles [38, 39]. In addition, we inferred that inner bulge cells had earlier expression patterns of the marker genes *Krt6a* [40] and *Krt10* [41], and developed earlier than outer bulge cells, about which there have been few reports [42]. On the whole, our study revealed the function of intermediate cells 10 in promoting the growth of cashmere and maintaining its attachment to the skin.

For cell fate 2, we enriched the Foxo-mediated transcription, p53 signaling pathway, PID REG GR pathway, and maintenance of location in cell. In particular, the p53 signaling pathway revealed the apoptotic process of cell fate 2. In addition, *krt6a* and *krt10* indicated that apoptosis in inner bulge cells was not only earlier than outer bulge cells, but also occurred faster and was more thorough. It is worth mentioning that we obtained a large number of pseudogenes in geneset2, and contrary to previous views, we believe that they are useful. The process of apoptosis involves both the regulation of normal gene expression and the competitive inhibition caused by the combination of pseudogene transcripts and the genome,

thus inhibiting the expression of normal genes [43, 44]; they are similar to lncRNA in function. Our results support the competitive endogenous RNA (ceRNA) hypothesis.

For cell fate 3, we enriched the apoptotic signaling pathway, Rab protein signal transduction, PID DELTA NP63 pathway, PID P73 pathway, PID BETA CATENIN NUC pathway, PID P53 DOWNSTREAM pathway, ERAD pathway, and PID MTOR 4 pathway in geneset1, which further revealed the function of the middle cell 1 in hair follicle apoptosis in cell fate 2. In addition, we enriched the TNFR2 non-canonical NF-kB pathway in geneset2, Wnt signaling pathway, signaling by NOTCH, and MAPK6/MAPK4 signaling, to reveal the function of intermediate cells 0 in hair follicle development [45, 46]. These two intermediate cells revealed a crosstalk process of apoptosis and development during hair follicle transition [47]. It is amazing that one type of cell controls two processes of apoptosis and development at the same time. For cell fate 4, we enriched the crosstalk process of multiple signaling pathways, illustrating the complexity of hair follicle in function. We enriched the maintenance of cell numbers, cellular communication, and regulation of cell morphogenesis during differentiation and maintenance of stem cell populations. Furthermore, in the pseudotime ordering result of cell fate 4, a part of the intermediate cells 0 transited into the intermediate cells 1 in the later stage, indicating apoptosis in these cells, further revealing that the number of DP cells in the hair follicle is relatively constant. This is consistent with previous studies that have observed that the number of DP cells determines the type of hair [17].

Overall, our study resolved the function of DP cells during transition through the hair follicle cycle, depicting four intermediate cells and their functions. In addition, we hypothesized the sequence of apoptosis in different bulge cells and the possible functions of pseudogenes in biological processes. In summary, this study provided new insights into the function of DP cells in cashmere goats during the transition of hair follicles from telogen to anagen and these data offer a deeper understanding of the molecular mechanisms of the cashmere cycle.

Methods

Experimental Animals

The study used 24-month-old female Inner Mongolian cashmere goats from Jinlai Animal Husbandry Technology Co., Ltd. All experimental procedures designed in this study have been approved by the experimental animal management committee of Inner Mongolia Agricultural University.

Histological Analysis And Immunofluorescence Staining

Tissue blocks, isolated from the dorsal skin were fixed with 4% paraformaldehyde (Leagene, Beijing, China) at 4 °C overnight. The next morning, the fixed tissues were dehydrated in an ethanol solution and further incubated with xylene for 30 min. After incubation with xylene the samples were embedded in paraffin blocks. The embedded paraffin blocks were cut with a Leica RM2235 microtome (Leica,

Nussloch, Germany) at a thickness of 5–7 μm and the samples were transferred to APES (ZSGB-BIO, Bejing, China) treated slides to avoid detachment.

For hematoxylin and eosin (H&E) staining, the slides were deparaffinized in 100% xylene solutions for 30 min and further rehydrated in an ethanol series. After rehydration the slides were stained with hematoxylin solution for 7 min followed by washing twice with distilled water for 5 min. After rinsing with 1% HCl (v/v) ethanol solution for 3–5 s, the slides were immediately washed with 45 °C water for 5 min. Slides were dehydrated and then stained with 1% eosin ethanol solution and further rinsed with absolute ethanol solution for 10 min. Finally, the slides were mounted with neutral resin mounting medium and pictures were taken under an optical microscope.

For immunofluorescence staining analysis, we used the bond-RXm fully automated immunohistochemistry system (Leica Microsystems GmbH, Wetzlar, Germany), pal ™ 7-Color Manual IHC Kit 50 slides reagent (PerkinElmer, USA). First each Opal Fluorophore was reconstituted in 75 uL of DMSO. Before each procedure, dilute Opal Fluorophore in 1X amplification diluent at 1:100 was used to make an Opal Fluorophore working solution. The primary antibody dilution ratios were: Lef1 (1:500), Lum (1:500) and CD34 (1:800). Samples were baked in an oven at 65° C for 1 h and transferred to the bond-RXm system (Supplementary Table 5, and antibody information can be seen in Table 1). Pictures were taken under a Leica TCS SP5 II confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Single Cell Suspension Preparation

For each group, skin tissues were obtained from at least three females. To prepare the dorsal skin single cell suspension for single-cell RNA sequencing, the dorsal skin tissues were isolated through microdissection and a 0.25% trypsin/EDTA solution was used for tissue digestion at 37^{O}C for 30 min. Samples were mechanically dissociated once every 10 min. An aliquot of 1 mg/ml collagenase IV (Sigma, St Louis, MO, USA) was used to digest the tissue with trypsin at 37^{O}C for 30 min. The skin tissues were mechanically dissociated into a single cell suspension by pipetting. Cell suspensions were then filtered through a 30 μm nylon cell strainer, to remove secondary hair debris (BD Falcon, BD Biosciences, San Jose, CA, USA) prior to single cell library construction.

Single Cell Library Preparation And Sequencing

Single cell barcoding and library preparation were performed based on the 10x Genomics single-cell RNA sequencing platform (10x Genomics, Pleasanton, CA, USA). Briefly, the single cell suspensions prepared above were immediately counted using a hemocytometer (TC20, Bio-Rad, Hercules, CA, USA) and the cell concentrations were adjusted to 1000 cells/μl prior to barcoding. To barcode the single cells with 10x Barcoded gel beads, the 10x Genomics Chromium Single Cell 3’ Library and Gel Bead Kit v2 (10x Genomics Inc., Pleasanton, CA, USA, 120237) and the 10x Genomics Chromium barcoding system was used to construct a 10x barcoded cDNA library following the manufacturer’s instructions. An Illumina

HiSeq X Ten sequencer (Illumina, San Diego, CA, USA) was used for sequencing and pair-ended 150 bp (PE150) reads were generated for downstream analysis.

10x Sequencing Data Preprocessing

CellRanger (v2.2.0) software was used for analyzing raw sequencing data according to the 10x Genomics official pipeline (https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/what-is-cell-ranger). Briefly, the sequencing raw base call (BCL) files were firstly transformed into FASTQ files using the “cellranger mkfastq” function. The generated FASTQ files were then processed with “cell ranger count” wrapped function with the “–force-cells = 7000” argument to adjust sample size. Cell ranger count function used wrapped STAR software to align sequence to the reference genome. The output files containing gene expression matrices and barcode information of the CellRanger pipeline were then used for downstream visualization analysis.

Characterization Of Cell Clusters

Following CellRanger pipeline, quality control (QC) and cell clustering were analyzed with single-cell RNA seq Seurat software (v2.3.0) based on R environment (R version: 3.5.3, https://www.r-project.org/) following the online guide (https://satijalab.org/seurat/). We used “filtered_gene_bc_matrices” files generated by CellRanger as input files for Seurat. For each dataset, we firstly filtered cells with less than 200 unique detected genes and genes detected with less than 3 cells, then we used the “FilterCells” function to remove cells with a total number of detected genes (nGenes) less than 1750. After normalization, the variable genes for each dataset were calculated for downstream clustering assay.

To compare transcriptome profiles along three different developmental timepoints, we then merged three different datasets using the “RunMultiCCA” function implemented in Seurat. RunMultiCCA used a canonical correlation analysis to remove variation caused by sample source. After dataset alignment, we then performed a clustering analysis on the integrated dataset based on the t-distributed Stochastic Neighbor Embedding (tSNE) algorithms implemented in Seurat. To identify the genes specifically expressed in clusters, we used the Seurat implemented “FindAllMarkers” function to calculate cluster markers and tSNE identified cell clusters were annotated based on previously reported canonical marker gene expression.

To subcluster cell clusters of interest for in-depth analysis and/or downstream differentiation trajectory construction, we used the Seurat implemented “SubsetData” function to extract clusters of interest. The extracted subclusters were then re-run through the Seurat pipeline, which provides higher resolution for dissecting cellular heterogeneity among particular cell types.

Constructing Single Cell Pseudotime Differentiation Trajectory

To interpret cell fate differentiation decisions, we used Monocle (v 2.4.0) to order single cells along pseudotime according to the official tutorial (http://cole-trapnell-lab.github.io/monocle-release/docs/#constructing-single-cell-trajectories). To perform pseudotime ordering on particular cell types, we firstly subclustered interested cell types from the Seurat object, then, the Monocle object was constructed using the “newCellDataSet” function in Monocle. To order single cells along pseudotime, we used Seurat identified variable genes as ordering genes to construct single cell differentiation trajectories. The root state was set according to cell Seurat identified cell cluster labels and the “BEAM” function was used to calculate branch-specific expressed genes. To plot a branch-specific expression heatmap, we used the Monocle implemented “plot_genes_branched_heatmap” function and genes with qval < 1e-4 were regarded as input genes. Gene clusters were further divided into four clusters according to k-means. To investigate gene functions in each gene cluster, we used Metascape (http://metascape.org/gp/index.html#/main/step1) to perform gene ontology (GO) analysis [48].

For Western blot analysis, we diluted the extracted protein 20 times and diluted the BSA standard to prepare the standard protein. After denaturation, the protein was separated by electrophoresis, and then β - actin (1:5000) and LEF1 (1:1000) were transferred to the membrane for 90 min at 200 mA. Finally, color development exposure was carried out by an ECL color development system, and the gray value of the film was analyzed by image-pro-plus.

Declarations

Data availability

The single cell RNA sequencing data used in this research is deposited in NCBI GEO databases under accession number: GSE141284.

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Ethics approval and consent to participate

All experimental procedures designed in this study have been approved by the experimental animal management committee of Inner Mongolia Agricultural University.

Consent for publication

All authors of the manuscript have read and agreed to its content and are accountable for all aspects of the accuracy and integrity of the manuscript in accordance with ICMJE criteria.The article is original, has

not already been published in a journal, and is not currently under consideration by another journal.And we agree to the terms of the BioMed Central Copyright and License Agreement.

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Declaration of Interests

The authors declare no competing interests including financial and non-financial.

Author contributions

FY and ZL made substantial contributions to the conception and design of the experiments and conceived and designed the experiments. CX, TG, and FY performed the experiments. FY analyzed the data. FY and ZL wrote the paper, QL, SZ, JW, YZ, XZ, GL, JZ, and RS critically revised the manuscript. All authors read and approved the final manuscript.

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List Of Abbreviations

The list of abbreviations	
Dermal papilla	DP
competitive endogenous RNA	ceRNA
single cell RNA	scRNA
t-distribution random neighbor embedding	tSNE
hair follicle in telogen	HFT
hair follicle in anagen	HFA
Single RNA	sRNA
quality control	QC
Gene Ontology	GO
Kyoto Encyclopedia of Genes and Genomes	KEGG

Supplementary Figure Legends

Figure S1: Quality control of single-cell data. (A) Single cell dataset quality metrics summary identified by CellRanger. (B) left, Violin plot displaying the number of genes (nGene), UMI (nUMI), and percentage of mitochondrial genes (percent.mito) detected in all single cells from three different datasets. The gene to UMI relationship for each dataset is also shown. Generally, the more UMI that was captured, the more genes were detected; right, average expression of genes. (C) left, The relationship between the number of unique molecules and the number of genes captured; middle, sum of gene expression; right, cell distribution in different periods.

Figure S2: Cell population identification and marker gene specific analysis. (A) Identification of different cell clusters by marker genes. Dermal cell markers: Lum, endothelial cell marker: Pecam1, matrix cell marker: Gata3; immune cell marker: Fcer1g; bulge cell marker: Sox9; melanocyte cell marker: Plp1; DP cell markers: Lef1, Msx1, Hoxc13, Pcna. (B) Notes for specific cell types. (C) Dot plot depicts representative cell marker gene expression. The dot size represents the percentage of cells expressed and the color intensity represents the relative expression level.

Figure S3: Overview of marker gene expression. (A) Expression of DP cell marker genes in all cell clusters. (B) Create interactive plots indicate DP cell location information. (C) Heatmap displaying all signature gene expressions in each cluster.

Figure S4: Cell state transition analysis. (A) All cell state transition and branch analysis. (B) DP cell state transition and branch analysis.

Figure S5: Overview of gene expression in all DP cells. (A) GO Biological Processes or KEGG Pathway Description. (B) Overlap between gene lists in left and genetic interaction in right. (C) GO function and KEGG signal path heat map in left and interaction between functional and signal path in right.

Figure S6: Protein interaction in geneset1, geneset2, geneset3, and geneset4

Figure S7: Function analysis of branch point 4. (A) KEGG Pathway Description. (B) Describes the difference of gene expression in pseudotime ordering of DP cell fate and cell fate 1.

Figure S8: Function analysis of branch point 2. (A) KEGG Pathway Description. (B) Describes the difference of gene expression in pseudotime ordering of DP cell fate and cell fate 2.

Figure S9: Function analysis of branch point 3. (A) GO Biological Processes or KEGG Pathway Description. (B) genes and their function in different genesets of cell fate 3. (C) Description of the difference of gene expression in pseudotime ordering of DP cell fate and cell fate 3. (D) Protein interaction network of all genesets. (E) Genetic interaction network of all genesets. (F) Protein interaction network of geneset1, geneset2, and geneset3.

Figure S10: Function analysis of branch point 1. (A) GO Biological Processes or KEGG Pathway Description. (B) GO function and KEGG signal path heat map. (C) Protein interaction network of all

genesets. (D) Genetic interaction network of all genesets. (E) Protein interaction network of geneset1, geneset2, and geneset3.

Figures

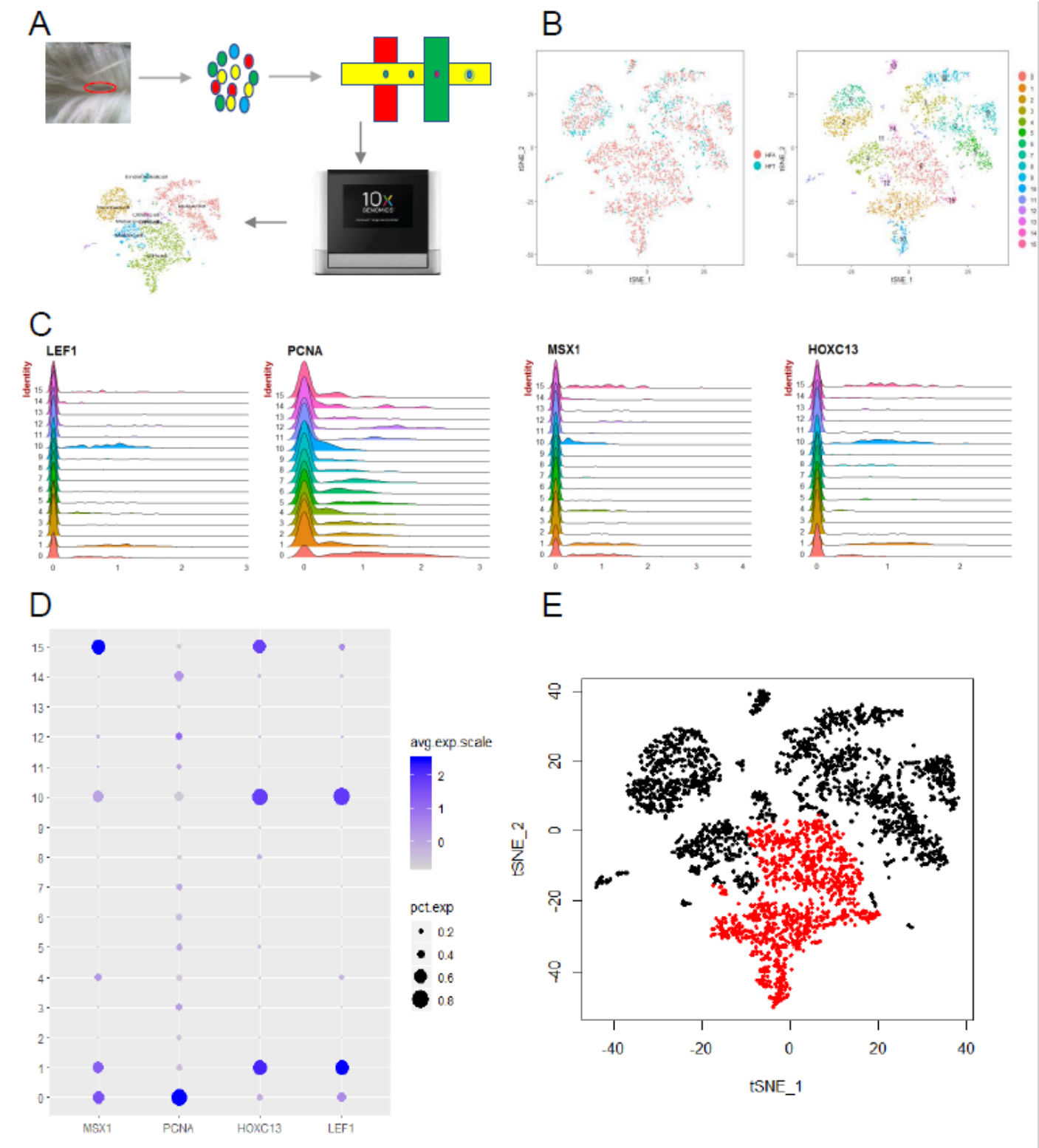

Figure 1

Overview of experimental procedures and characterization of major cell populations from skin tissues. (A) Schematic diagram illustrating the experimental pipeline for scRNA seq analysis of cashmere goat skin tissues. Single cell transcriptomes were obtained based on the 10x Chromium platform. (B) tSNE clustering of single skin cells. Each point represents one single cell and cells in the same cluster have similar transcriptome profiles. To the left is the tSNE plot of the integrated dataset from 3 different time points and cells are color-coded according to developmental time point. The right plot depicts 15 transcriptional distinct cell clusters and cells are color-coded with cluster information. (C) Different marker gene expression across all DP cells in the tSNE plot. (D) Dot plot depicts representative DP cell gene expression. The dot size represents the percentage of cells expressed and the color intensity represents the relative expression level. (E) tSNE clustering of single skin cells, red dots represent the extracted DP cell population, and black dots represent other cell populations.

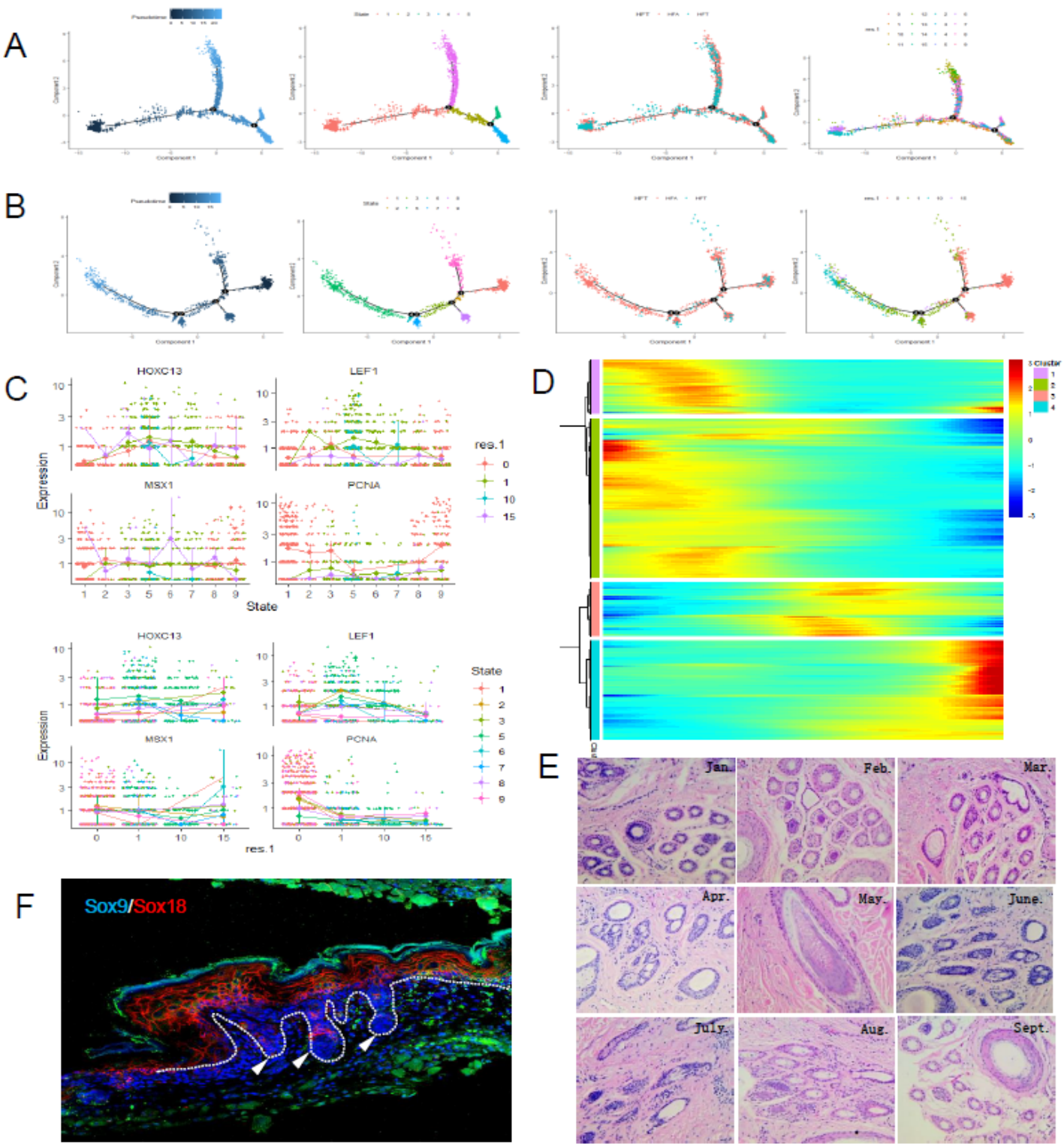

Figure 2

Summarizes the function of DP cells on hair follicle development. (A) pseudotime ordering of all cells, (B) pseudotime ordering of DP cells, (C) overview of expression of DP marker genes, (D) DP cell gene expression pattern heat map, (E) January–September skin tissue H&E staining, (F) immunofluorescence analysis of sox9 and sox18 expression in the embryonic skin.

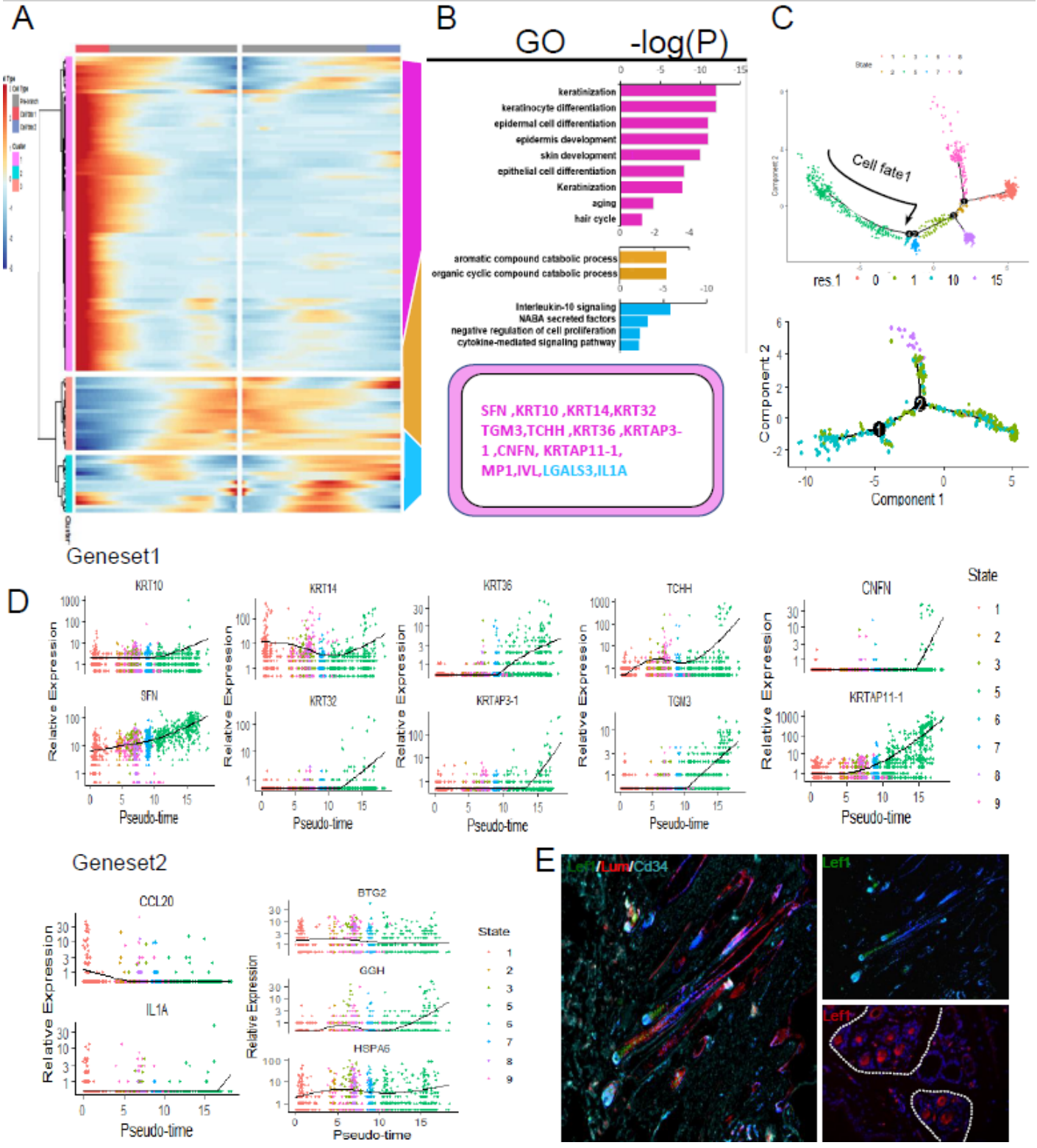

Figure 3

Recapitulating intermediate cell 10 fate decision towards maintenance of hair follicles and secondary hair. (A) Heat map showing the specific DEG expression pattern of cell fate 1 in Figure 3C. (B) Enriched GO function and high expression gene visualization. (C) Pseudotime ordering analysis with DP cells on top and cell fate 1 on the bottom. (D) Visualization of the transition of highly expressed genes in

pseudotime ordering of DP cells. (E) Immunofluorescence analysis of Lef1 expression in the skin of prophase telogen.

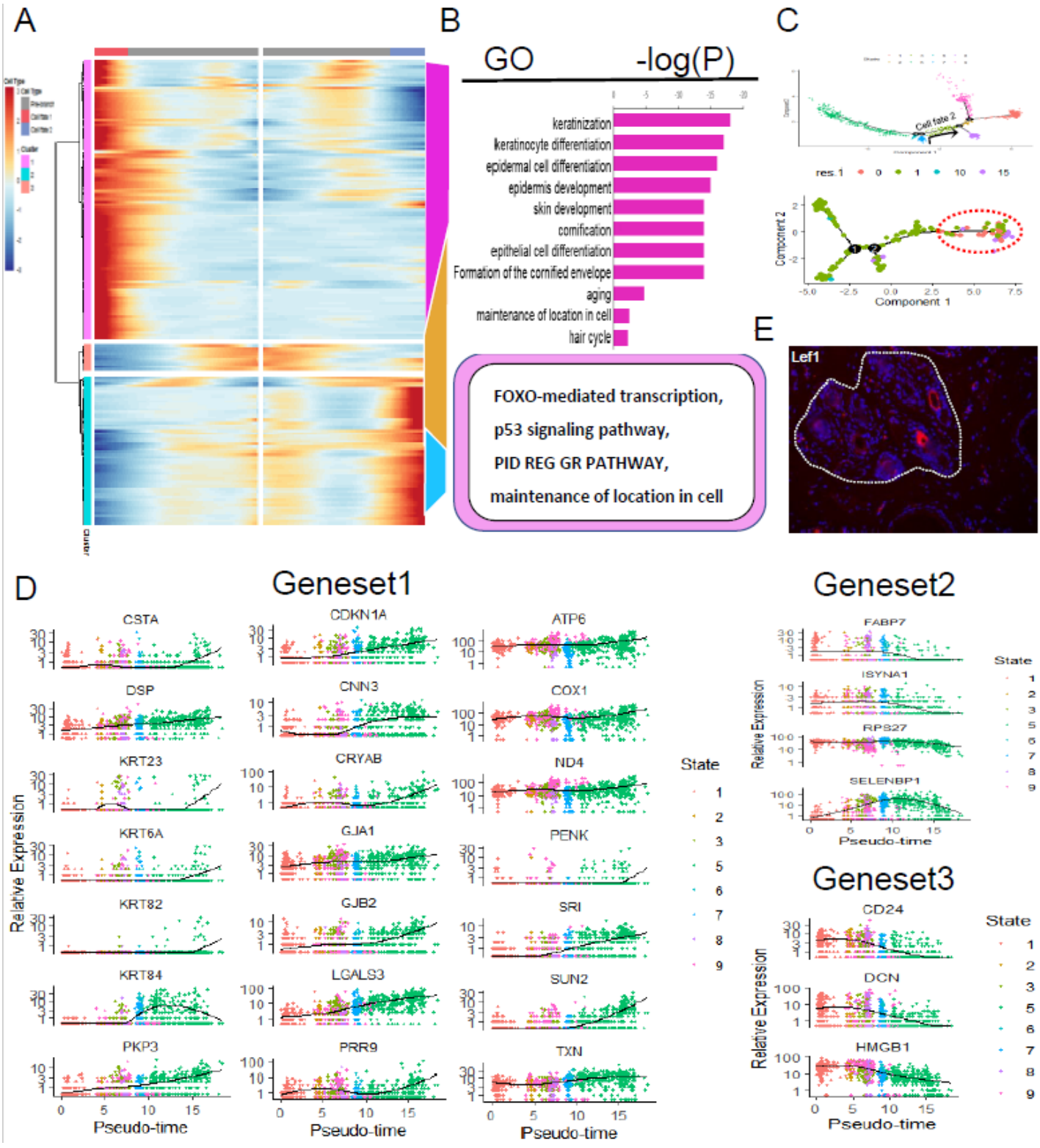

Figure 4

Dissecting intermediate cell 1 fate decision towards apoptosis of hair follicles. (A) Heat map showing the specific DEG expression pattern of cell fate 2 in Figure 4C. (B) Enriched GO function and KEGG signaling pathway. (C) Pseudotime ordering analysis with DP cells on top and cell fate 2 on the bottom. (D)

Visualization of the transition of highly expressed genes in pseudotime ordering of DP cells. (E) Immunofluorescence analysis of Lef1 expression in the skin of later telogen.

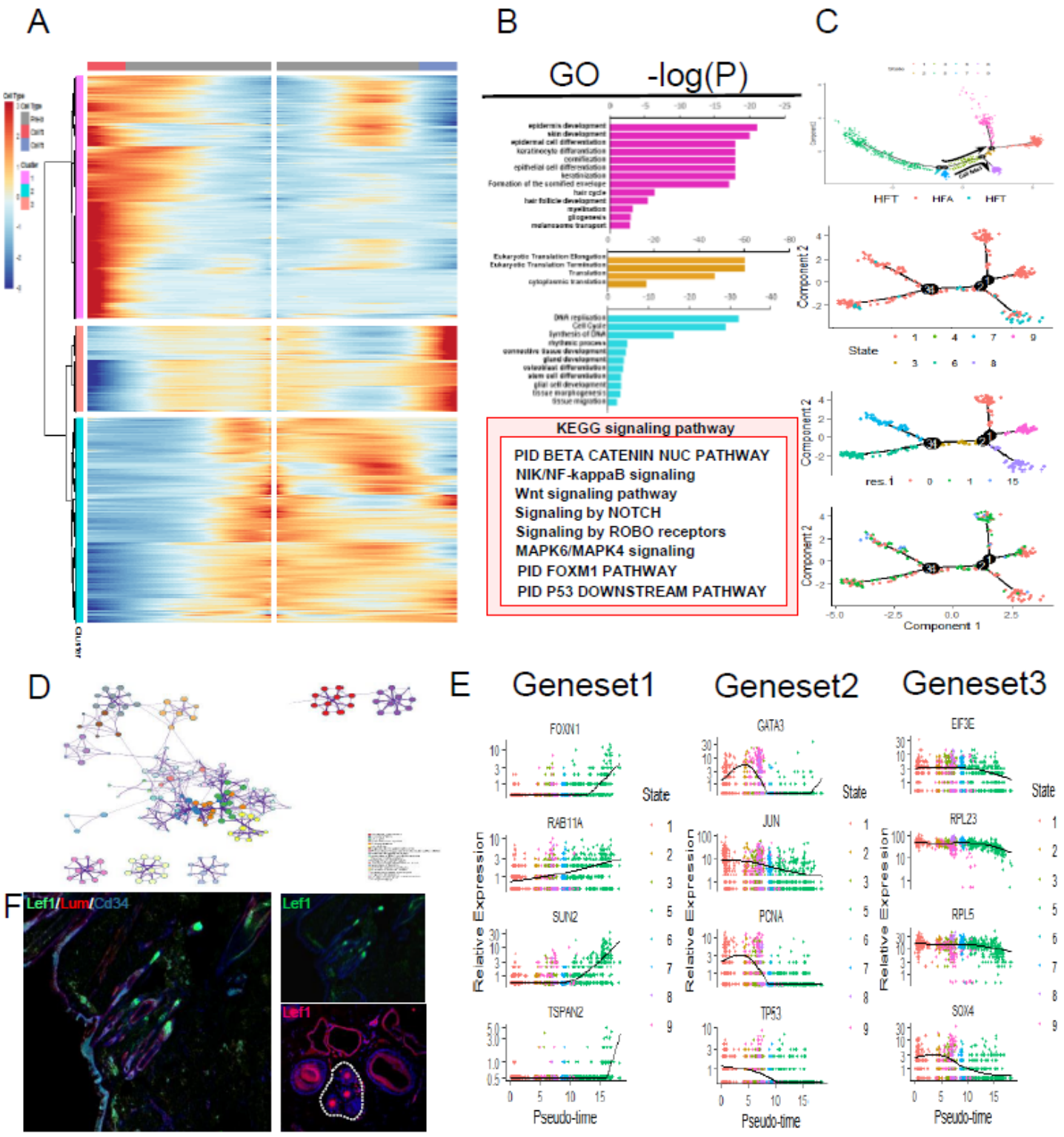

Figure 5

Describing the crosstalk process of old hair follicle apoptosis and new hair follicle development. (A) Heat map showing the specific DEG expression pattern of cell fate 3 in Figure 5C. (B) Enriched GO function and KEGG signaling pathway. (C) Pseudotime ordering analysis with DP cells on top and cell fate 3 on

the bottom. (D) Interaction network diagram of GO functions and KEGG signaling pathways. (E) Visualization of the transition of highly expressed genes in pseudotime ordering of DP cells. (F) Immunofluorescence analysis of Lef1 expression in the skin of later telogen and prophase anagen.

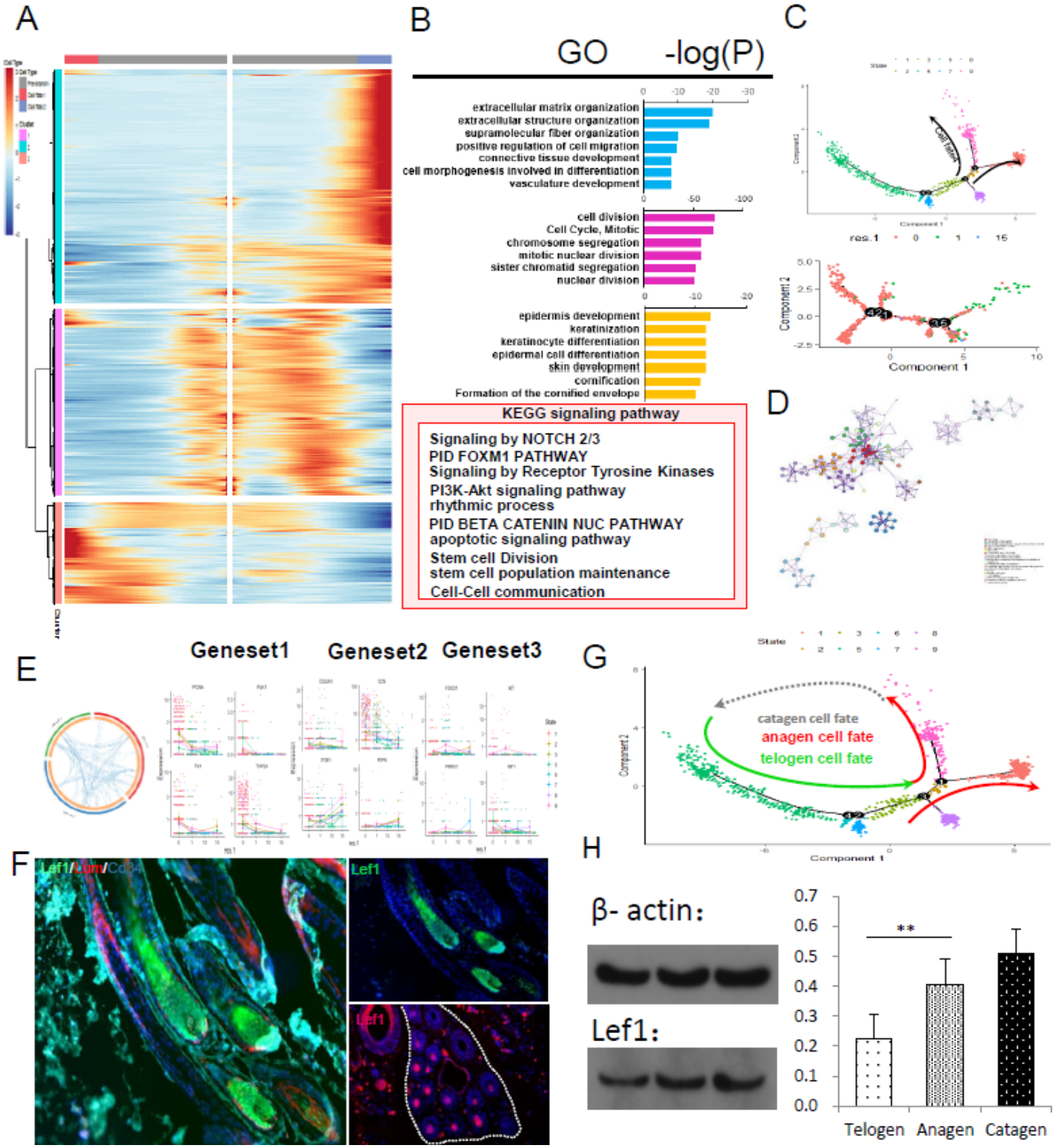

Figure 6

Revealing intermediate cell 0 fate decision towards development of hair follicles. (A) Heat map showing the specific DEG expression pattern of cell fate 4 in Figure 6C. (B) Enriched GO function and KEGG

signaling pathway. (C) Pseudotime ordering analysis with DP cells on top and cell fate 4 on the bottom. (D) Interaction network diagram of GO function and KEGG signal path. (E) Overlap between gene lists and visualization of the transition of highly expressed genes in pseudotime ordering of DP cells. (F) Immunofluorescence analysis of Lef1 expression in the skin of middle & late anagen. (G) DP cell fate decisions towards hair follicles in telogen, anagen and catagen.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfigure.pdf
- SupplementaryTable4.xlsx
- SupplementaryTable5.pdf
- SupplementaryTable2.xlsx
- SupplementaryTable1.csv
- SupplementaryTable3.xlsx