

# Notch1 down-regulation in lineage-restricted niches of mouse eccrine sweat glands

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## Research

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# **Abstract**

## Background

Eccrine sweat gland (SG) restrictedly exists in mouse foot pads indicating that mouse plantar dermis (PD) contains the SG lineage-restricted niches. However, it is still unclear how niches can affect stem cell fates.

## Methods

In this study, we tried to find the key cues by which stem cells sense and interact with the SG lineage-specific niches. Briefly, we used transcriptomics RNA sequencing analysis to screen differentially expressed genes between SG cells and epidermal stem cells (ES), and then we used proteomic analysis to screen differentially expressed proteins between PD and dorsal dermis (DD).

## Results

We found that Notch1 is not only closely related to embryonic SG morphogenesis based on Gene Ontology enrichment analysis but also differentially down-regulated during SG formation in the levels of genes and proteins. Furthermore, immunochemistry and immunofluorescence staining verified that Notch1 was continuously down-regulated along with the process of SG morphogenesis. Especially, Notch1 positive cells almost disappeared neither in the emerging SG buds or in the newly-formed glandular structures.

## Conclusion

Hence, we speculated that Notch1 possibly acts as the role of “gatekeeper” during embryonic SG development and is the promising key cue that regulates the interactions between stem cells and the SG lineage-specific niches. Our attempts highlighted the role of Notch1 during embryonic SG organogenesis.

## Trial registration

Not applicable.

# **Introduction**

Eccrine sweat gland (SG) originates from the keratin (K) 14 positive embryonic epidermal stem cells (ES) [1] acting as the most abundant glands of human being and playing important roles in thermoregulation and metabolism [2]. Interestingly, eccrine SG restrictedly exists in mouse foot pads [1]. In other words, ES committed SG lineage in mouse foot pads but differentiated into other lineages (i.e., hair follicles or sebaceous glands) in other body positions [3]. The fact that ES committed SG fates only in mouse foot pads indicated that mouse plantar dermis (PD) probably serves as SG lineage-restricted niches.

A niche is anatomically and functionally defined as a lineage-restricted microenvironment [4] which directly maintains stem cell self-renewal and regulates cellular activities [5]. Recent progress highlighted the roles of niches in carcinogenesis [6], committing stem cell lineages [7] as well as regulating the morphogenesis of tissue or organs [8]. Stem cell lineages were committed by both their intrinsic nature and the extrinsic features endowed by niches [9]. For example, hematopoietic and muscle stem cells have remarkable plasticity depending on environmental stimuli [10]. Additionally, both the neural stem cells [11] and the adult testicular cells [12] were able to transdifferentiate into mammary glands when engrafted into cleared mammary pads of immunocompromised mice. Therefore, a niche sometimes gets the ascendancy over the intrinsic natures of stem cells in directing their fates.

Since microenvironments can greatly impact stem cell behaviors, whether the niches in mouse PD can redirect the fates of other stem cells into SG? To explore that, Ferraris et al [13] mixed rabbit corneal epithelium together with embryonic mouse PD, dorsal dermis (DD) or upper-lips, respectively, and then grafted these recombinants under the kidney capsule of athymic nude mice. Results showed that SG were only found in the PD-contained recombinants, while hair follicles together with pilosebaceous units were regenerated in both the DD-contained and upper-lips-contained recombinants [13]. Also, our team recently proved that mouse PD successfully directed the fates of ES [14] and redirected mesenchymal stem cells [15] or mammary progenitor cells [16] toward SG in a 3D-bioprinted hydrogel matrix. It seems that stem cells are able to not only “remember” their original lineages but also “respond to” the new microenvironment and possibly “change” their lineages.

How niches can affect stem cell fates? Generally, stem cells interact with niches via mechanical cues (i.e., matrix stiffness, applied forces, et al.) [17] or biochemical cues (i.e., signaling pathways, growth factors, ions, extracellular matrix, cytokines, et al.) [18] during embryonic development. However, it remains largely unexplored what are the key cues by which stem cells sense and interact with the SG lineage-specific niches.

Here, we aimed to screen these key cues that played a leading role in the interaction between stem cells and the SG lineage-specific niches. Differentially expressed proteins or genes involved in SG morphogenesis were regarded as the promising candidates based on transcriptomic RNA sequencing analysis, proteomic analysis and Gene Ontology enrichment analysis. Also, immunochemistry and immunofluorescence staining were used for further verifying the changes of the candidate(s) during SG development. Our work might be helpful for understanding mechanisms of embryonic SG organogenesis and possibly for tailoring artificial niches for SG regenerative therapies.

## Materials And Methods

### Mice

C57BL/6 mice were purchased from Sibeifu Company (Beijing, China). Although the time-depended processes of embryonic mouse SG morphogenesis have been reported [19, 20], individual conditions of each mouse vary greatly. Moreover, day ages of mouse embryos were ambiguously marked in some

reports. In order to precisely know the embryonic day ages, we put male and female mice together at night and then separate them in the next morning. Once the female mice were later found to be pregnant, this time point was marked as day 0.5. Pregnant female mice were caged separately without any unnecessary treatments.

All mice were caged in low humidity environment with food and tap water ad libitum. All animal procedures were approved (approval number SCXK(BJ)2019-0001) and supervised by Institutional Animal Care and USE Committee of Chinese PLA General Hospital (Beijing, China).

### **Histological study of SG in foot pads**

Embryonic day 12.5 (E12.5), embryonic day 17.5 (E17.5), postpartum day 1 (P1), postpartum day 5 (P5), postpartum day 14 (P14) aged mice or embryos were sacrificed for studying the developing process of SG. Briefly, foot pads were harvested following sterilizations by immersed into 75% alcohol for about 30 min and then were fixed in 4% paraformaldehyde solutions for up to one day. In order to remove calcium in bones, these foot pads were immersed for two days in decalcifying solutions (Solarbio, G2470) containing formic acids, formaldehyde, ethylene diamine tetraacetic acids (EDTA), etc. The foot pads were then embedded in paraffin and were sectioned 6- $\mu$ m thick by using a cryotome (Leica, CM1950, Germany) following with H&E staining procedures.

### **RNA sequencing analysis of SG cells (P1) and ES (E12.5)**

#### **a. Harvesting SG cells (P1)**

The foot pads of newborn mouse (P1) were cut up into pieces about 5mm\*5mm, which were then immersed into 2 mg/ml Collagenase I solutions with occasionally shaking at 37 °C for about 2 hours. After that, SG cells could be found on the cross section of dermal regions using a phase contrast microscope. We isolated SG cells from adjacent tissues and aspirated them up using sterilized micropipettes under direct vision. These cells were cultured in a cell incubator using SG cell specific medium, which contains 50% F12 (STEMCELL) and 50% DMEM (STEMCELL) basal culture medium supplemented with final concentrations of 0.4 mg/ml hydrocortisone succinate (Gibco), 2 ng/ml liothyronine sodium (Gibco), 1% insulin-transferrin-selenium (Gibco), 10 ng/ml epidermal growth factor (Peprotech, Rocky Hill, NJ) as well as 1% penicillin-streptomycin solutions and 5% fetal calf serum (Gibco).

#### **b. Harvesting ES (E12.5)**

Briefly, the skin was immersed into 2 mg/ml Dispase solutions overnight, and the epidermis was carefully separated from the skin sample with tweezers, during which the epidermis cannot dry thoroughly. Then, the epidermis was cut into small pieces and was immersed into 0.05% EDTA-contained trypsin with occasionally shaking to achieve fully digestion at 37 °C for about 20 min. A cell strainer (FALCON, 40 mm Nylon) was used to remove tissue fragments with larger sizes. The epidermal cell suspensions were collected following centrifuge under sterilized conditions with relative centrifuge force of 400g for 5 min.

The precipitates were resuspended with Keratinocyte Serum Free Medium (GIBCO, 10724-011) and were then seeded onto cell culturing dishes for following tests. Cell culturing or amplification were necessary to get rid of unwanted cells, such as fibroblasts, blood cells or vascular endothelial cells. After cultivating for several generations, the remaining cells, which were regarded as ES with pluripotency abilities, acted as a control group of SG cells for the following test.

### c. RNA sequencing analysis

Total RNA of SG cells (P1) and ES (E12.5) was extracted using TRIzol (Invitrogen). RNA sequencing analysis was performed with HiSeq 2500 (Illumina). Genes with fold difference > 2.0, mean log intensity > 2.0 and false discovery rate < 0.05 were considered to be significant.

### Proteomics analysis of PD (P1) and DD (P1)

Briefly, dermal tissues of foot pads and dorsal trunk of were homogenized with isotonic phosphate buffer (pH 7.4) with final concentration of 25% (w/v) following a centrifuge at 10,000g for 20 min at 4°C. The supernatants were then collected and were frozen at -80 °C for proteomics analysis. All procedures were conducted in an ice bath for reducing protein degradation and under sterilized conditions for avoiding contamination of intruding microbial proteins. The proteomics analysis was involved of using isobaric tags for relative and absolute quantification (iTRAQ) in BGI Company.

### Immunohistochemistry staining of Notch1

All sections were deparaffinized and rehydrated. After incubation in 0.3% hydrogen peroxide solutions, the sections were then boiled in 0.01 M citric acid buffer (pH 6.0) for about 10 min following slowly cooling down to room temperature for antigen retrieval. In order to block nonspecific antigen sites, sections were incubated with 5% goat serum diluted with PBS at room temperature for about 1 h. Sections were permeabilized with 0.3% Triton X-100 (Sigma-Aldrich, USA) for about 15 min and were incubated with Notch1 antibody (Rabbit mAb, CST#3608, 1:500) at 4°C overnight. After rinsing slides with PBS, biotinylated goat anti-rabbit immunoglobulins as secondary antibody was incubated following counterstaining with Mayer's hematoxylin as well as dehydration and coverslips.

### Immunofluorescence double staining of Notch1 and K14

As mentioned above, sweat buds emerge from the keratin 14 (K14) positive progenitors [1], we used immunofluorescence double staining of Notch1 and K14 to explore how Notch1 changes during the developing process of SG. Briefly, sections were incubated with the mixture of Notch1 antibody (Rabbit mAb, CST#3608, 1:500) and SG marker K14 (Mouse mAb, Abcami#7800, 1:300) at 4°C overnight. After rinsing, the sections were incubated with the mixture of Alexa Fluor 594 goat-anti-rabbit (1:300) and Alexa Fluor 488 goat-anti-mouse (1:300) as secondary antibodies for 2 hours at room temperature in dark. Finally, sections were incubated with 4',6-diamidino-2-phenylindole (Beyotime, Beijing) for 20 min and were visualized under a confocal microscope.

## Results

# The process of mouse SG morphogenesis

Mouse SG are restrictedly distributed in paw pads at the side of palms. The paw pads are a small amount of tissues bulged out the skin of palms, near to which are located with more tissues, such as tendons, nerves and blood vessels (Fig. 1). E12.5 is almost the earliest time point that mice were found pregnant judged by our naked eyes. SG haven't yet emerged at E12.5 (Fig. 1A). SG buds, a group of branching epitheliums, emerged at about E17.5 from the basal layers of paw pads (Fig. 1B). SG ducts stretched from the buds at about P1 (Fig. 1C) and extended toward deeper dermis. Glandular structures emerged at about P5 (Fig. 1D). Mature SG with secreting functions were formed until about P14 (Fig. 1E).

Our histological studies are consistent with published reports that SG buds emerge just before birth [1] and canonically complete structures formed at postpartum day 0 to 3 (P0 ~ P3) [19]. Therefore, PD and DD for proteomics analysis were both harvested from P1 mice. As for RNA sequencing analysis, SG cells were harvested from P1 mice, while the ES, the control groups for RNA sequencing analysis, were harvested from the skin of mouse embryos' trunk at a very early embryonic stage (E12.5) to make sure that SG haven't yet emerged (Fig. 1F) [21].

### Notch1 and laminin alpha 5 (Lama5) were down-regulated both in SG cells and PD

Firstly, we performed three Gene Ontology terms involved in embryonic SG development (i.e., stem cell differentiation, morphogenesis of a branching epithelium, and gland morphogenesis) to elucidate the transcriptomic differences between SG cells and ES as detected by RNA sequencing. Heat maps of relatively expressed genes involved in these three Gene Ontology terms showed that SG cells and ES exhibited much different transcriptomic profiles (Fig. 2A).

Secondly, we believed that those differentially expressed genes are promising candidates of key cues that are involved in directing ES to commit SG lineages. Based on this idea, we totally screened out 12 genes that are not only involved in SG morphogenesis but also differentially expressed in SG cells and ES: Bmp4, Bmp7, Lrp5, Msx2, Notch1, Lama5, Sfrp1, Shh, Sox9, Tfap2c, Tgfb1, Wnt4 (Fig. 2B).

Thirdly, we asked whether the 12 genes have translated correspondingly differential amount of proteins because proteins act as the main executors in the life processes and directly participate in regulating cellular activities. Results showed that only Notch1 and laminin alpha 5 (Lama5) were differently expressed between PD and DD as detected by proteomics analysis. Interestingly, both Notch1 and Lama5 were significantly down-regulated not only in gene expression (Fig. 2C, E) but also in protein levels (Fig. 2D, F) at P1.

Reportedly, Lama5 is one type of the laminin proteins, which are composed of alpha, beta and gamma chains and act as the major components of basement membranes [22]. Lama5 is located in cell nucleus and is broadly expressed in lung, kidney, skin, neural tube, limb and many other tissues and organs [23].

Although Lama5 has shown to affect cellular activities of human umbilical vein endothelial cells [24] and the mutations of Lama5 can cause bone dysplasia [25] or affect the differentiation of colorectal cancer cells [26], no convincing evidences about Lama5 regulating SG morphogenesis have yet to be found. Therefore, we highly suspect that Notch1 acts as the key cues which mainly regulated the fates of ES toward SG lineages.

### **Notch1 was down-regulated along with the process of SG morphogenesis**

As RNA sequencing and proteomics analysis were performed at only one time point (P1), we then asked whether Notch1 was down-regulated along with the whole process of SG morphogenesis. To verify that, immunohistochemistry staining of Notch1 and immunofluorescence double staining of Notch1 and K14 were performed at crucial time points (i.e., E12.5, E17.5, P1, P5) during SG development.

Results showed that Notch1 was highly and widely expressed at skin basal layers, dermis and blood vessels at E12.5 (Fig. 3A, 4A). Along with the emerging of SG buds at E17.5, Notch1 significantly decreased all over the dermis, especially in basal layers (Fig. 3B, 4B). The overall distribution of Notch1 continuously decreased at P5 (Fig. 3D, 4D). Interestingly, Notch1 positive cells almost disappeared neither in the SG buds at E17.5 (Fig. 3B, 4B) or in the newly-formed glandular structures at P1 (Fig. 4C). There are still a few Notch1-K14-double-positive cells located in the basal layers and deeper dermis both at E17.5 (Fig. 4B) and P5 (Fig. 4D).

## **Discussion**

Except for regulating body temperature and excreting electrolytes and water, eccrine SG can form a stratified interfollicular epidermis acting as an additional source of keratinocytes for skin self-renew and wound healing [27]. Therefore, SG regeneration promisingly acts as a crucial milestone for perfect wound healing with restored appendages and reduced scars. However, the regeneration of eccrine SG based on stem cell therapies has always been challenging difficulties.

One of the insurmountable obstacles of SG regeneration is the intractability of stem cells behaviors endowed by surrounding microenvironments [28]. Previous study showed that once mouse sweat duct progenitors were transplanted into the shoulder fat pads of nude mice, the SG-like structures were successfully *de novo* regenerated, indicating that sweat glandular progenitors can faithfully maintain their identity even when placed within foreign microenvironments or niches [29]. However, the fate of these glandular progenitors and the morphogenesis of SG were strongly depended on local microenvironments or niches. For example, mouse sweat duct progenitors were transplanted into the cleared mammary fat pads of virgin or lactating nude mice, respectively. Results showed that although SG-like structures were regenerated in both groups of mice, about 20% of *de novo* glands in lactating nude mice displayed typical mammary gland-like morphogenesis characterized by branched and enlarged lumens accompanied by milk protein expression [29]. In this study, some sweat duct progenitors responded to the changes of hormones in local microenvironments and changed their fates

correspondingly. Therefore, SG lineage-specific stem cells are highly environmental-depended and are very sensitive to external stimuli, which making SG regeneration so difficult after injuries [28].

How did SG lineage-specific stem cells sense or interact with their local environment? In this study, we found that Notch1 is closely related to SG morphogenesis based on Gene Ontology analysis (Fig. 2B). Notch1 was not only down-regulated in SG cells based on the RNA sequencing analysis (Fig. 2C) but also down-regulated in PD based on the proteomics analysis (Fig. 2D). Particularly, we didn't find Notch1 positive cells neither in the SG buds at E17.5 (Fig. 3B, 4B) or in the newly-formed glandular structures at P1 (Fig. 4C). Although a few Notch1-K14-double-positive cells was reserved in the basal layers or deeper dermis at E17.5 (Fig. 4B) and P5 (Fig. 4D), a continuous downtrend of Notch1 was verified along with the process of SG morphogenesis based on the immunohistochemistry and immunofluorescence staining (Figs. 3 and 4). The Notch1-K14-double-positive cells are probably the residual progenitor cells with multipotent or unipotent abilities. Hence, we speculated that Notch1 possibly acts as the role of "gatekeeper" during embryonic SG development and is the promising key cue that regulates the interactions between stem cells and the SG lineage-specific niches.

The Notch receptor was firstly discovered in *Drosophila melanogaster* a century ago acting as an evolutionarily conserved pathway that widely regulates cell fates through local cell communications [30]. In mammals, the Notch signaling systems include four paralogous receptors (Notch 1 to Notch 4) and the transmembrane ligands (Delta-like-1, Deltalike-2, Delta-like-4, Jagged-1 and Jagged-2). Notch signaling act as the sensor and instructor to combine multiple biochemical and physical extracellular inputs from the local microenvironment to coordinate cell fate and organ morphogenesis [31]. Previous studies reported that Notch signaling systems regulate hypertrophic scar formation after wound healing [32], hair follicle morphogenesis [33] as well as the multipotency of glandular stem cells, such as mammary glands [34], prostate glands [35] or salivary glands [36]. However, it remains undocumented whether Notch1 acts as the key cue via which SG lineage-specific stem cells sense or interact with their niches.

Our study highlighted the role of Notch1 during embryonic SG organogenesis. The spatiotemporal down-regulation of Notch1 in SG lineage-specific niches suggested that Notch1-involved signaling pathways played important roles in regulating embryonic SG organogenesis, and artificially blocking or knocking out Notch1 might trigger the SG morphogenesis or repair the dysfunction of SG after injury. In the near future, we also look forward to the Notch1-signal-tunable "artificial niches" aiming to control stem cell fates and to elucidate regulatory mechanisms during SG regeneration [37]. Hopefully, remarkable prospects of tailored stem cell fates have been shown by the "artificial niches" in terms of tuning critical signaling pathways [38] or mimicking key components of the endogenous niches [39].

## Conclusion

In this study, we screened the differentially expressed proteins and genes both in SG lineage-restricted niches and in progenitor cells during SG morphogenesis. Results indicated that Notch1 was the promising key cue that regulates the interactions between stem cells and the SG lineage-specific niches.

The continuous down-regulation of Notch1 possibly suggested that Notch1 acts as the role of “gatekeeper” during embryonic SG development. However, whether tuning Notch1-related signaling pathways benefits SG regeneration should be further verified.

## Abbreviations

DD  
dorsal dermis  
E  
embryonic day  
ES  
epidermal stem cell  
K  
keratin  
Lama5  
laminin alpha 5  
P  
postpartum day  
PD  
plantar dermis  
SG  
sweat gland

## Declarations

### Ethical Approval and Consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and materials

All the data and materials in this study are available from the corresponding author upon reasonable request.

### Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Authors' contributions

Yuzhen Wang proposed the detailed plan of this research, carried out Gene Ontology enrichment analysis, immunochemistry staining and immunofluorescence staining, and wrote the manuscript. Bin Yao provided step-by-step instructions and performed RNA sequencing analysis and proteomic analysis. Xianlan Duan, Jianjun Li, and Wei Song harvested and cultured SG cells and ES. Enhejirigala, Zhao Li, Xingyu Yuan, Yi Kong, and Yijie Zhang provided help in making figures and analyzing data. Xiaobing Fu and Sha Huang provided the most funding fees, supervised the whole process of research and edited the manuscript.

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## Figures

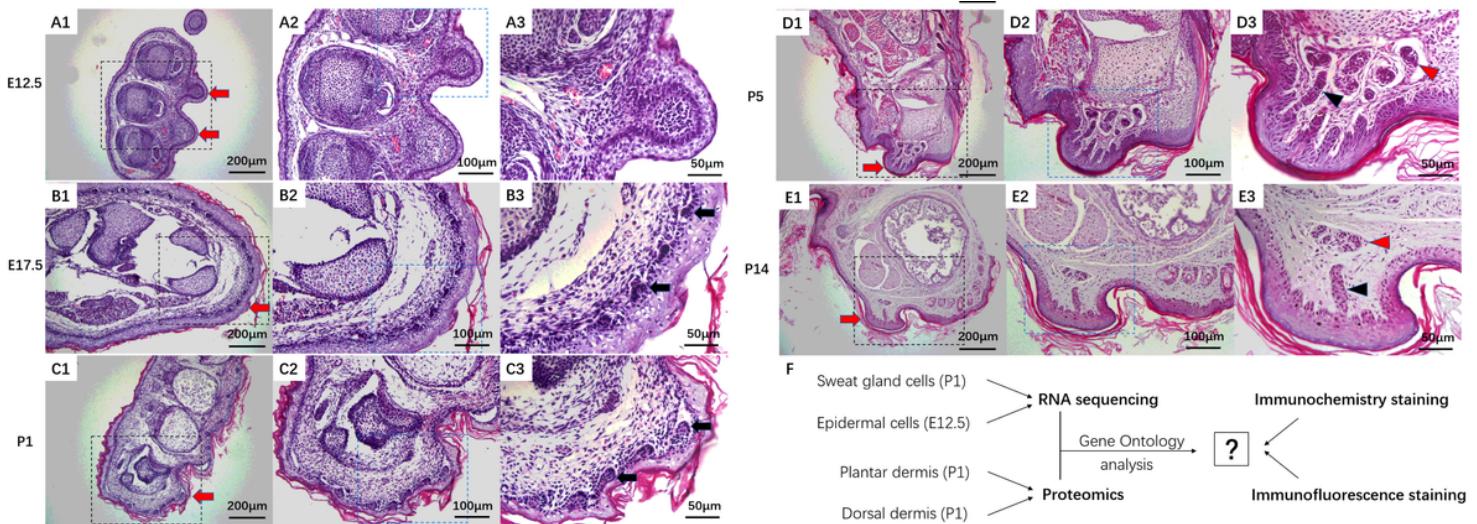
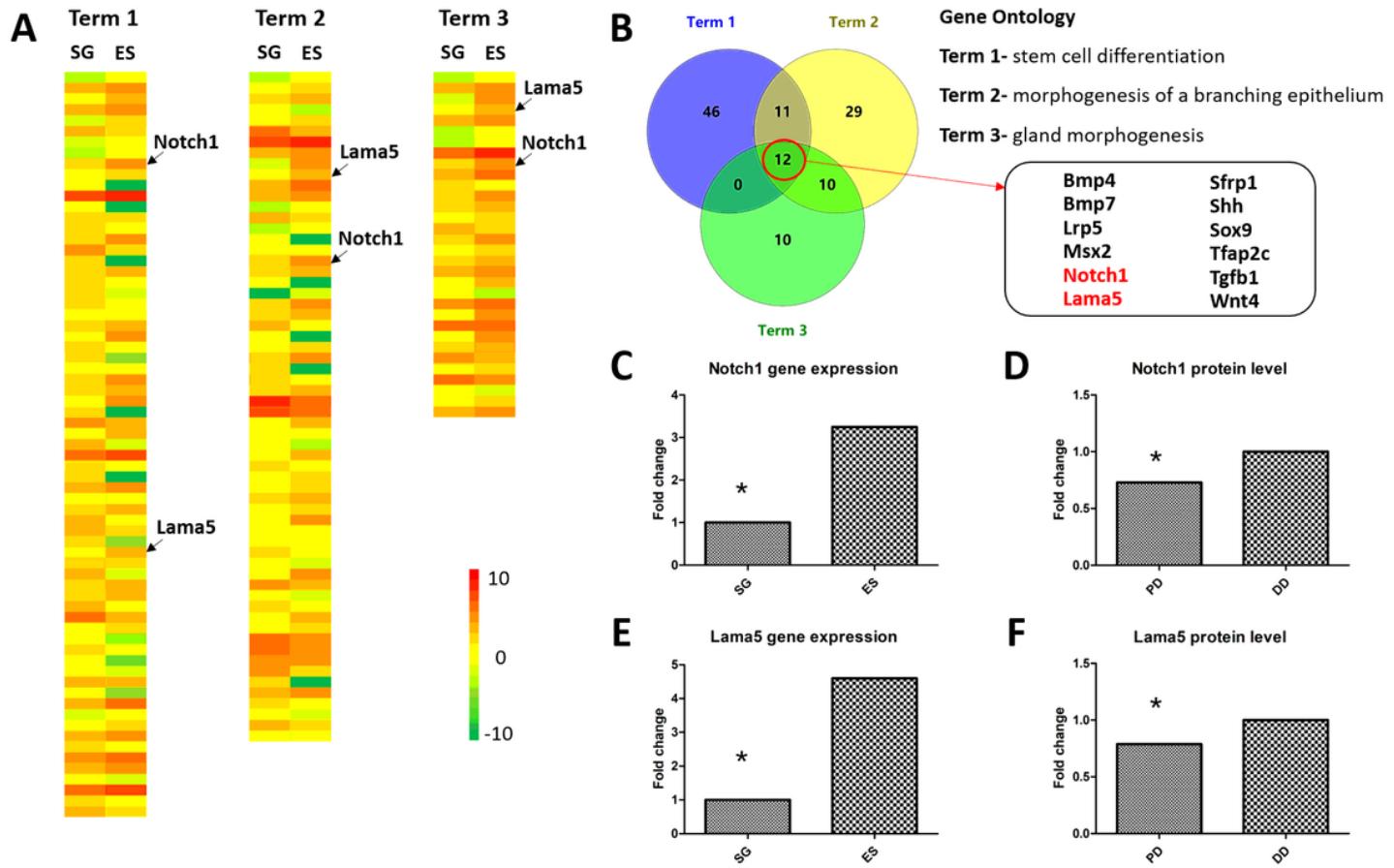


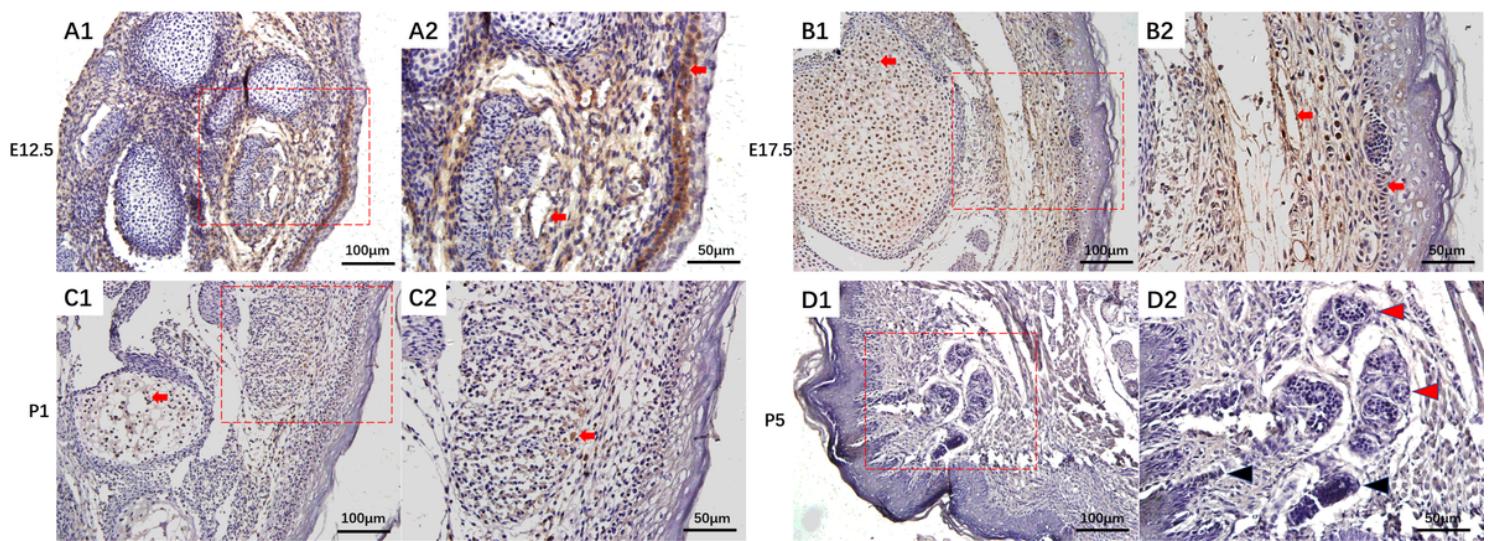
Figure 1

The embryonic morphogenesis of mouse SG and the schematic illustration of this study. H&E staining of mouse foot pads are shown at different time points: E12.5 (A1~A3), E17.5 (B1~B3), P1 (C1~C3), P5 (D1~D3), P14 (E1~E3). The schematic workflow of our study was shown in (F). Red arrows indicate foot pads. Black arrows indicate SG buds (B3) or the emerging SG ducts (C3). Red triangles indicate glandular structures. Black triangles indicate SG ducts. Rectangles highlight zoom area on the right.



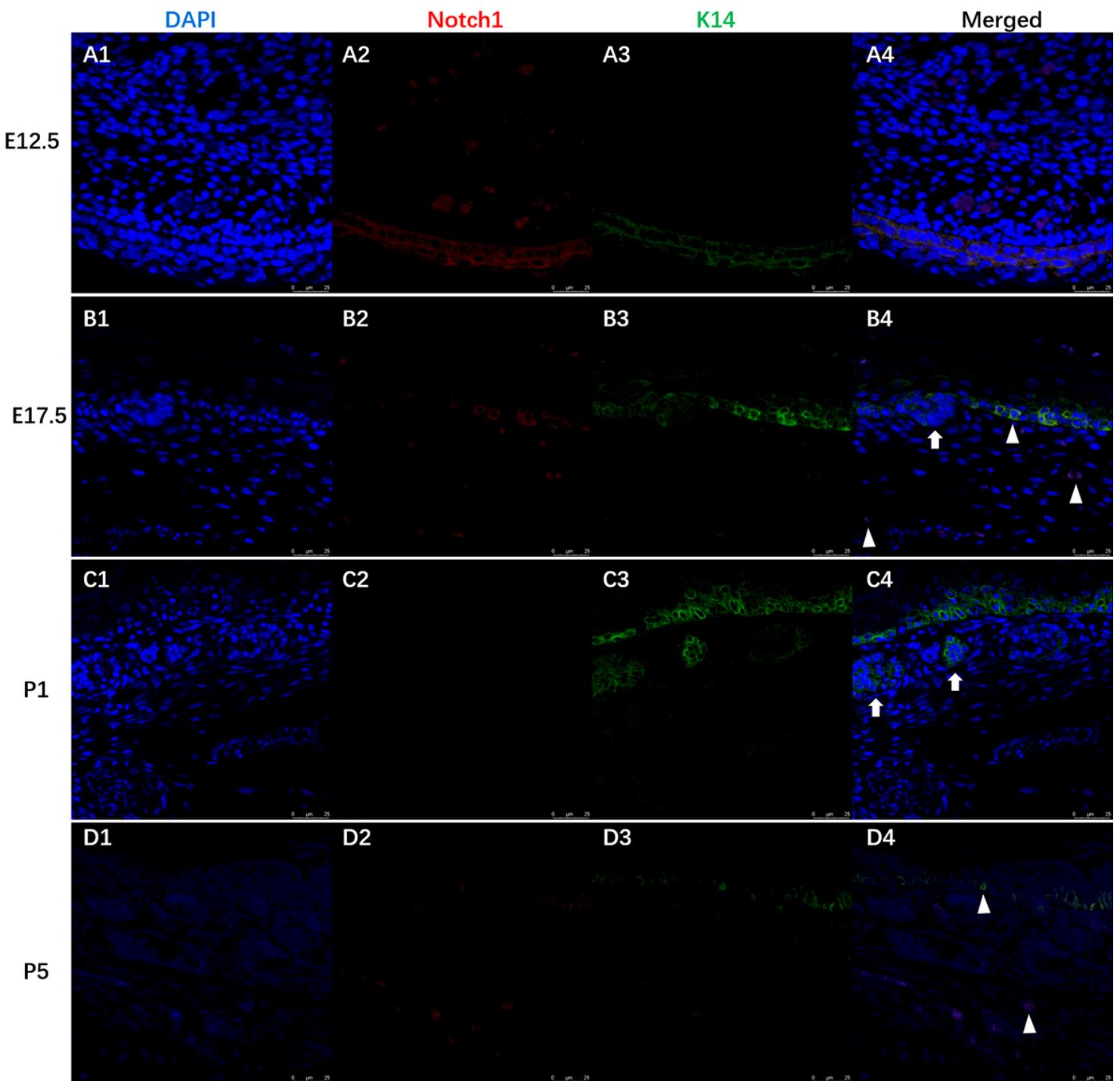
**Figure 2**

Notch1 and Lama5 was down-regulated both in SG cells and PD. (A) Heat maps illustrating differential expression of genes involved in embryonic SG development between SG cells and ES. (B) Three Gene Ontology terms (i.e., stem cell differentiation, morphogenesis of a branching epithelium, and gland morphogenesis) were used to screen differentially expressed genes. Totally, 12 genes were screened out in a Venn map. The gene expression of Notch1 (C) and Lama5 (E) between SG and ES based on RNA sequencing analysis. The protein level of Notch1 (D) and Lama5 (F) between PD and DD based on proteomics analysis. Abbreviations: SG: sweat gland; ES: epidermal stem cell; PD: plantar dermis; DD: dorsal dermis; Lama5: laminin alpha 5.



**Figure 3**

Immunohistochemistry staining of Notch1. Notch1 was down-regulated along with the whole process of SG morphogenesis at E12.5 (A1~2), E17.5 (B1~2), P1 (C1~2), P5 (D1~2), respectively. Red arrows indicate Notch1 positive cells. Red triangles indicate glandular structures. Black triangles indicate SG ducts. Rectangles highlight zoom area on the right.



**Figure 4**

Immunofluorescence double staining of Notch1 and K14. Notch1 was down-regulated along with the whole process of SG morphogenesis at E12.5 (A1~4), E17.5 (B1~4), P1 (C1~4), P5 (D1~4), respectively. White arrows indicate SG buds (B4) or glandular structures (C4). White triangles indicate the Notch1-K14-double-positive cells (B4, D4). DAPI, blue; Notch1, red; K14, green; scale bars: 25 μm.