Exploration of Key Signals in Alopecia Areata by Cell-Cell Interaction Analysis

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***Abstract*—Alopecia Areata (AA) is an autoimmune disease resulting from a breach in the immune privilege of the hair follicles. To gain insights into the mechanism, we focused on the interactions between cells in the hair follicles. Dermal Papilla (DP) cells are especially essential for developing hair follicles and maintaining the growth phase of the hair cycle. We used a single-cell RNA sequencing dataset of hair follicles to analyze the interaction of immune cells, primarily with DP cells. This dataset was obtained from a patient with AA. To identify cell types that interact with DP cells and contribute to AA, we performed clustering of fibroblasts and T cells to identify DP and T cell subtype populations. Through cell-cell communication analysis, we also identified DP as the most statistically frequent interacting cell type. In addition, we identified T cell subtypes that interact with papilla cells and ligand-receptor pairs. By integrating the results of these analyses, we inferred specific T cell subtypes that interact with DP cells and predicted novel ligand-receptor pairs that shorten the growth (anagen) phase of the hair cycle by causing inflammation in both follicular and DP cells.**

***Keywords*—cell-cell interaction analysis, single-cell RNA sequencing, dermal papilla, hair follicle, T cell, alopecia areata**

I. Introduction

Hair is an important feature of mammals, which performs a wide range of functions, including thermoregulation, body protection, sensory activity, and social interaction [1]. Hair is an organ derived from the epidermis and is divided into an external and internal structure. The outer part of the skin is composed of epithelial cells that are dead and completely keratinized. In contrast, the part buried inside the skin is a structure of living cells called the hair follicle. The dermal papilla (DP), derived from the mesenchyme, and the keratinocytes surrounding it form the hair bulb [2].

The hair follicle is a skin appendage and consists of structures such as DP cells. DP cells are located at the base of the hair, adjacent to the Dermal Sheath (DS), a DP precursor cell, and the sheath-like keratinocytes surrounding the hair [1]. Hair growth and hair loss are regulated by the hair follicle cycle. There are three main phases of the hair follicle cycle: anagen (growth), catagen (regression), and telogen (rest). Hair continues to grow during the anagen phase, followed by a transitional period of the catagen phase, which enters into the telogen phase when hair is released from the follicle and falls [3].

Alopecia Areata (AA) is an autoimmune form of hair loss characterized by atrophy and shrinkage of hair follicles and papilla and infiltration of lymphocytes [4]. Immune cell attack induces inflammation in the hair follicles of patients with alopecia areata. As a result, the anagen phase of the follicle is shortened and the follicle enters the catagen phase [5]. Therefore, for hair regeneration, it is necessary to maintain the anagen by activating the DP and suppressing inflammation caused by immune cells.

In this study, we aimed to analyze the interaction between DP and cells in the hair follicle, mainly immune cells, to detect the ligand-receptor pairs associated with alopecia areata. Our method consists of the following steps: (1) Clustering analysis and cell type identification, (2) Identification of DP cell populations and T cell subtype populations, (3) Detection of source and target by cell-cell communication analysis, (4) Cell-cell communication analysis to detect ligand-receptor pairs, (5) Detection of most significant interactions between the cell types.

For Step (4), we introduced a method to statistically compare ligand-receptor expression differences in multiple conditions (AA patients and healthy controls). Conventional cell-cell communication analysis focuses on the visual comparison of expression differences of ligand-receptor pairs in multiple conditions; however, we recognized that it is difficult to identify statistical significance between the conditions. To address this issue, our study employs a method that detects expression differences of ligand-receptor pairs and statistically compares these differences using the Wald test statistics for ranking. This method enables us to clearly detect differential expression between multiple conditions. This allows for more precise analysis by capturing differences between conditions, even in the case of subtle expression differences. In addition, our study highlights the most frequent targets in AA patients and analyzes the differential expression of their ligand-receptor pairs. This

Manuscript received September 9, 2024; accepted November 6, 2024; published June 20, 2025.

will identify cell-cell interactions that may play a critical role in the pathogenesis and provide new insights into disease mechanisms. Focusing on the most frequent targets provides a novel perspective beyond the simple evaluation of individual ligand-receptor pairs to address the core aspects of pathology.

II. Literature Review

Several studies have shown that interactions between cells within the hair follicles are related to AA and development. Ober-Reynolds *et al.* [6] generate single-cell transcriptome datasets and identify diverse cell types in scalp tissue from healthy controls and patients with AA. This study identifies gene regulatory networks in healthy and diseased hair follicles using single-cell gene expression and chromatin accessibility datasets. However, they do not fully investigate the interactions between DP and surrounding cells.

Additionally, they did not examine which types of immune cells trigger inflammation in hair follicles and DPs. In addition, immune cells have been shown to play an important role in hair growth and follicle development. Lee *et al.* [7] generated single-cell transcript datasets from the skin tissue of healthy controls and AA patients to identify specific subtypes of immune cells. However, the interaction of DP with surrounding immune cells has not been fully investigated. It has also not been investigated which subtypes of immune cells cause inflammation in the hair follicle and DP.

III. Materials and Methods

A. Single-Cell RNA-seq of Hair Follicles from Healthy Controls and Patients with AA

We have conducted scRNA-seq analysis on hair follicles from both healthy individuals and patients with AA to elucidate the signaling pathways in the hair follicles centered around the DP. We analyzed AA using a scRNA-seq dataset obtained by Ober-Reynolds *et al.* (GSE212450) [6]. The dataset includes scRNA-seq results from human scalp tissue samples, comprising AA patients (AA, n = 5), healthy control volunteers (C_PB, n = 3), and surgical scalp tissue (C_SD, n = 7). For this study, we adopted C_PB and AA as we needed scRNA-seq datasets from both healthy and disease states of the hair follicles.

B. Clustering Analysis and Cell Type Inference

We clustered the scRNA-seq data of hair follicles to infer the Dermal Papilla (DP), which plays an important role in hair development, and T cells involved in the pathogenesis of Alopecia Areata (AA). However, DP and T cell subtypes are cell types that were not identified in the original paper of scRNA-seq data [6]. Therefore, we performed annotation using marker genes and inferred the cell types. As preprocessing, we performed quality control, normalization, and scaling on the data. We removed batch effects and integrated the data. Then, we performed clustering analysis on the integrated dataset. Based on the expression levels of marker genes, we inferred the cell types for the clusters. *COL1A1*, *COL1A2*, and *THY1* for fibroblasts; *CD3D* for T cells.

C. Identification of DP Cell Population and T Cell Subtype Populations

Next, we identified subtypes from the fibroblasts, which are a higher-level classification of DP cells, and from T cells, which play an important role in the autoimmune disease AA. Based on marker gene expression levels, we performed subclustering of the fibroblast clusters and inferred DP cells and Dermal Sheath (DS) as precursors of DP. Additionally, we subclustered and annotated the T cell clusters, identifying the following five subclusters: naïve T cells, central memory T cells (TCM), effector memory T cells (TEM), terminally differentiated effector memory T cells re-expressing CD45RA (TEMRA), and regulatory T cells (Treg). Annotation of the subclusters was performed using known marker genes from previous studies [6, 8]. *WNT5A*, *PTCH1*, and *HHIP* for DP cells; *SOX2*, *EDNRA*, and *NCAM1* for DS (precursors of DP); *PTPRC* for naive T cells; *CCR7* for TCM; *GZMA* for TEM; *CST7* for TEMRA; *FOXP3* for Treg as marker genes.

D. Detection of Source and Target by Cell-Cell Communication Analysis

To identify cell types and signals associated with AA, mainly DP, we performed cell-cell communication analysis among cell types obtained from scRNA-seq clustering analysis. As the next step, we conducted a cell-cell communication analysis among the cell type populations, which was identified through clustering analysis and annotation. This analysis involves studying the interactions between cells by inferring communication patterns through signaling molecules such as proteins and other molecules. First, we generated pseudobulk profiles for each cell type in the patient (AA) and healthy control (C_PB) datasets. Next, differential expression analysis between AA and healthy control conditions was performed. After generating feature statistics for each cell type, we linked them to a ligand-receptor resource and calculated the average feature expression of each cell type population and expression proportions. Subsequently, ligand-receptor pairs with an adjusted p-value < 0.05 were defined as statistically significant signaling molecules associated with AA. We calculated the distribution of the inferred pairs, where the cell types expressing ligands were designated as sources, and the cell types expressing receptors were selected as targets.

E. Cell-Cell Communication Analysis to Infer Ligand-Receptor Pairs

A comprehensive evaluation of the detected ligand-receptor pairs was performed in this step. Rather than focusing on specific sources or targets, we evaluated all identified ligand-receptor interactions to gain a broad understanding of the communication patterns between different cell types. This analysis aimed to evaluate the entire network of cell-cell interactions without focusing on specific sources or targets.

F. Detection of Most Significant Interactions between Cell Types

To identify the most impactful interactions, we focused on cell-cell communication patterns involving the most frequent. We extracted and prioritized ligand-receptor pairs that specifically targeted DP cells, as DP was identified as a significant target cell type in the previous analysis. This step aimed to highlight the most relevant interactions that may play a critical role in AA and related pathways.

G. Software and Tools Used

We used Seurat 4.3 for preprocessing and clustering analysis of the single-cell RNA-seq data [9]. Batch effects were removed using Harmony 1.0 [10]. For the cell-cell interaction analysis, we used LIANA+ as the method and ConsensusDB, a database that integrates multiple resources, as the database [11].

IV. Result and Discussion

A. Clustering and Cell Type Annotation

As a result of clustering, 24 distinct clusters were identified (Fig. 1). As a result of annotation with marker genes, 9 cell types were assigned to the clusters, enabling the identification of the fibroblasts and T cell clusters.

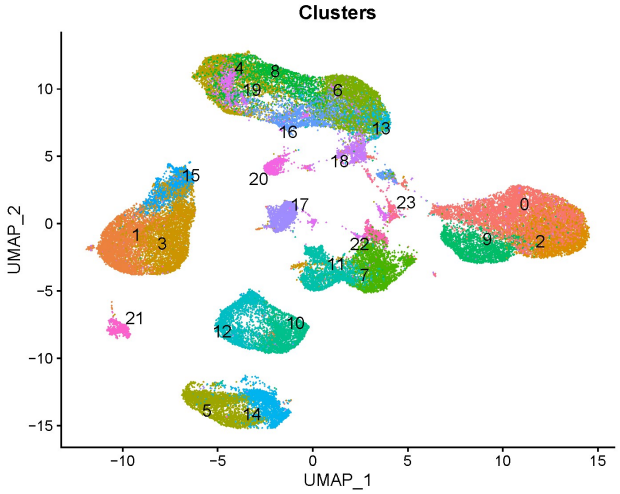

Fig. 1. UMAP embedding with clustering results.

B. Identification of DP Cell Population and T Cell Subtype Populations by Subclustering

Clusters 1, 3, and 15 exhibited high gene expression of the marker genes for fibroblasts. Subsequent subclustering identified 10 different subclusters including DP and DS (Fig. 2). Subclusters 4 and 6 showed high expression of the marker genes for DS and DP, respectively (Fig. 3). Clusters 0, 2, and 9 exhibited high expression of the marker genes for T cells. Subsequent subclustering identified 13 different subclusters including T cell subtypes (Fig. 4). Subclustering of the T cell cluster identified subclusters containing subtypes such as Naive T cells, TCM, TEM, TEMRA, and Treg (Fig. 5). Resulting whole cell type annotations are shown in Fig. 6.

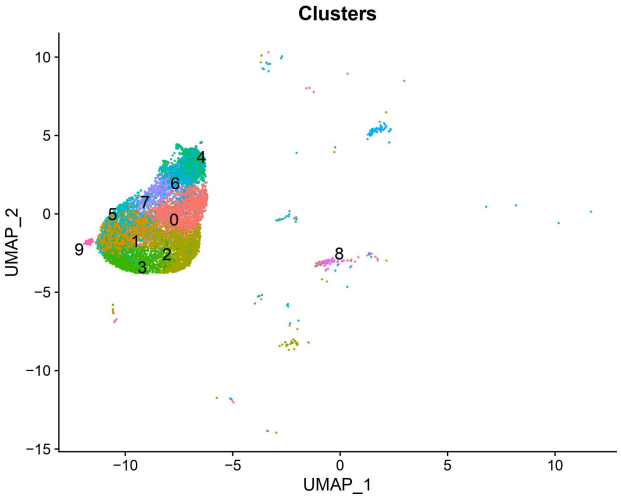

Fig. 2. UMAP embedding with subclustering results of the fibroblast population (clusters 1, 3, and 15).

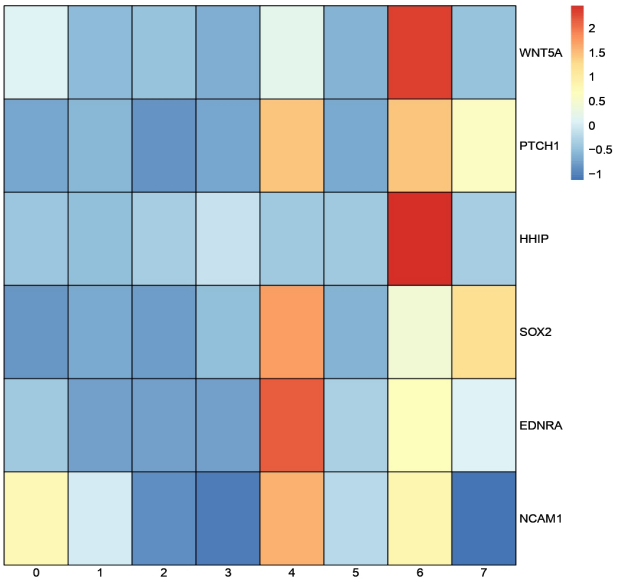

Fig. 3. Heatmap of marker gene expression in fibroblast subclusters. Subclusters 4 and 6 are annotated as DP and DS, respectively.

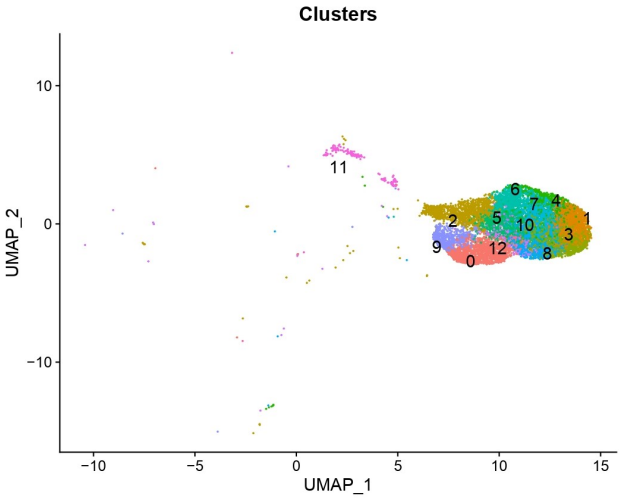

Fig. 4. UMAP embedding with subclustering results of the t cell population (clusters 0, 2, and 9).

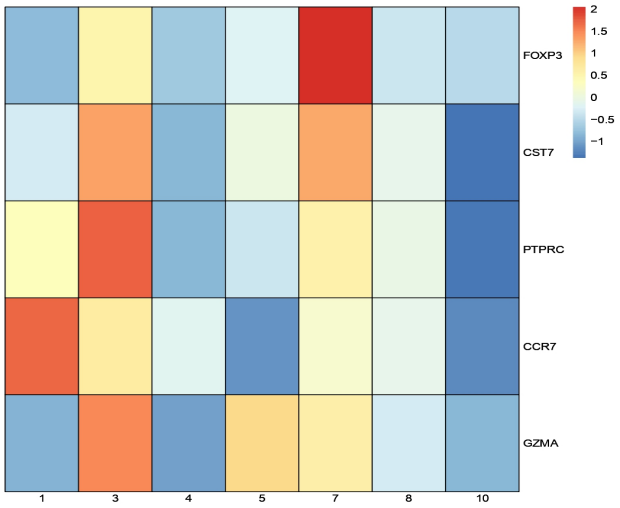

Fig. 5. Heatmap of marker gene expression in T cells subclusters. Subclusters 3, 1, 5, 8, and 7 are annotated as naive T cells, TCM, TEM, TEMRA, and Treg, respectively.

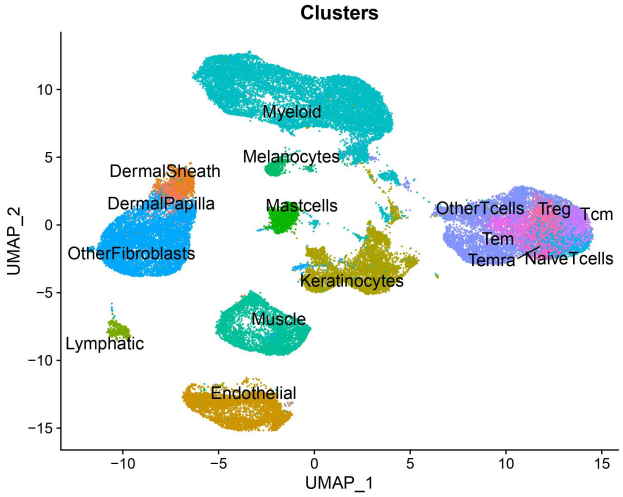

Fig. 6. UMAP embedding with clusters and subclusters results with cell type annotations resulted in 17 cell types.

C. Identification of AA-Related Interactions by Cell-Cell Communication Analysis

TABLE I. INTERACTIONS DETECTED BY CELL-CELL COMMUNICATION ANALYSIS IN AA. ALL INTERACTIONS ARE TARGETED TO DP. ADJUSTED *P*-VALUE < 0.05.

Ligand	Receptor	Source	Interaction stat.[1]	Interaction p-adj.[2]
CXCL12	*AVPR1A*	DP	5.24	5.42E-15
CXCL12	*AVPR1A*	DS	3.99	7.49E-07
CXCL12	*SDC4*	DP	3.84	5.42E-15
TGFB3	*ENG*	Endothelial	3.31	4.56E-05
TNFSF13B	*CD40*	Keratinocytes	2.75	4.27E-03
CXCL12	*SDC4*	DS	2.60	7.49E-07
DLL1	*NOTCH3*	Endothelial	2.49	2.62E-03
ADM	*CALCRL*	Lymphatic	2.46	3.98E-02
CCL5	*SDC4*	TEM	2.00	2.20E-04
ADM	*RAMP2*	Lymphatic	1.99	3.98E-02
BGN	*LY96*	Myeloid	1.96	7.48E-03
FSTL1	*DIP2A*	Endothelial	1.62	3.59E-02
ADAM12	*SDC4*	DS	1.52	6.21E-03
CCL5	*SDC4*	TEMRA	1.37	1.11E-02

[1] interaction statistic
[2] adjusted *p*-value.

Compared to C_PB, 46 ligand-receptor pairs associated with AA were detected. Among the detected pairs, there were 14 pairs with DP as the target, 9 with lymphatic, 8 with naive T cells, 6 with endothelial, 4 with myeloid, 2 with keratinocytes, and 1 each with muscle, other fibroblasts, and TEM. The most frequently detected ligand-receptor pairs targeting DP are shown (Table I).

D. Discussion

The purpose of this study was to identify hair follicle cell types and signal types that interact with DP cells to elucidate the mechanism of AA. Analysis of human follicle single-cell RNA-seq revealed T cell subtypes and signals that interact with DPs in the alopecia condition, suggesting their possible involvement in the mechanism.

Our method of analysis successfully inferred DP and T cell subtypes by clustering and identifying cell types by expression levels of marker genes. In addition, through cell-cell communication analysis, we were able to infer the signals between DP, which is essential for hair development, and T cell subtypes, which play a crucial role in the autoimmune disease AA.

In this study, we introduced a method to detect differences in the expression of ligand-receptor pairs and to compare these differences statistically (ranking by the Wald test statistics). This allowed us to compare the importance of the detected pairs with the statistics.

Furthermore, in this study, we focused on DP, the most frequent target in AA patients, and analyzed the differential expression of the ligand-receptor pairs. This identifies interactions between DP and other cells, which have not been extensively discussed in AA research, and provides new insights into the mechanisms of the disease.

In this study, 14 ligand-receptor pairs associated with AA were detected. These pairs target DP, which is essential for hair follicle development. Of the 14 pairs, 3 had DS or endothelial as the source, 2 had DP or Lymphatic, and 1 had keratinocytes, myeloid, TEM, or TEMRA. In the present study, no ligand-receptor pairs were derived from Treg or naive T cells, or TCM among the T cell subtypes. For example, Tregs also play a role in regulating immune responses and suppressing autoimmune reactions [12]. In AA, Treg function is reduced or dysfunctional, potentially leading to an exaggerated autoimmune response and attack on the hair follicle. Nour *et al.* [13] reported that increasing Tregs in the skin in a mouse model of alopecia resulted in hair regeneration and inhibition of inflammatory pathways. Additionally, it has been reported that in AA patients, the proportion of Treg cells is lower than other T cell types, while the inflammatory cytokine IL-17 increases in the serum. On the other hand, anti-inflammatory cytokines produced by Tregs, such as IL-12 and IL-35, decrease [14]. Therefore, it is possible that this study did not detect AA-related signals originating from Tregs due to the increased inflammatory signals from other T cells.

Of the AA-related interactions identified, 4 were pairs with *CXCL12* as the ligand. C-X-C Motif Chemokine Ligand 12 (CXCL12) is an immune-attracting factor called chemokine. In this study, we have confirmed for the first time that CXCL12 signaling is enhanced in DP

and DS of hair follicles from patients with AA. Previous studies have reported enhanced *CXCL12* signaling in dermal fibroblasts from mouse models of AA [15], but this is the first confirmation in humans. These findings suggest that enhanced CXCL12 signaling in hair follicles may be involved in the pathogenesis of AA.

There is no previous report in AA on the ligand and receptor pairs, *CCL5* and *SDC4* identified in this study. However, CCL5 is an IFN-ɤ-inducible chemokine and one of the signature genes of AA [16]. IFN-ɤ-inducible chemokines attract T and NK cells to inflammatory sites. Therefore, this signal may play a role in attracting other T cells to the DP, causing an excessive autoimmune response and progression of AA.

V. Conclusion

In conclusion, this study represents a cell-cell interaction analysis of hair follicle scRNA-seq to understand the DP-centered mechanism in AA.

Our analysis has identified a novel ligand-receptor pair, *CCL5* and *SDC4*, which attract immune cells such as T cells to the DP, cause inflammation, and may be involved in follicular fibrosis and loss of hair follicles.

This finding is to be investigated in future studies, enabling a more detailed characterization of the molecular basis of hair follicle degeneration in AA.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

AMI and HM conducted the research, analyzed the data, and wrote the paper; HS and SS provided critical feedback; all authors had approved the final version.

Funding

This work was supported in part by JSPS KAKENHI Grant Numbers JP21K19827 and JP24K01949.

Acknowledgment

AMI and HM have received research support from Nissin Foods Holdings Co., Ltd.

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