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Single-cell transcriptome analysis reveals characteristic transcription factors in polydactyly

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Abstract. – OBJECTIVE: The study aims to provide guidance on the identification of multiple-digit malformations as potential biomarkers and therapeutic targets.

MATERIALS AND METHODS: Single-cell RNA sequencing (scRNA-seq) data of four multiple-finger malformation samples were downloaded from the GEO public database. Fibroblasts and keratinocytes were divided into cellular subpopulations and the transcription factors of different subpopulations were analyzed. The regulatory network of transcription factors and their target genes were constructed to analyze the functionality of regulons.

RESULTS: Examination of the transcriptional profile data from 11,806 single cells uncovered significant associations between regulons and cell function in polydactyly. Specifically, the analysis highlighted the involvement of HOX family members and GLI2 transcription factors, including HOXD13, MSX2, LHX2, EMX2, LEF1, CREB3L2, and LHX2, in the polydactyly process within fibroblast cells. Furthermore, it sheds light on the roles of HES2 and GLIS1 in the formation and development of keratinocytes.

CONCLUSIONS: Significant presence of transcription factors, especially HOXD13, MSX2, and LHX2, may be strongly related to the development of polydactyly.

Key Words:
Polydactyly, Transcription factors, Single-cell transcriptome analysis, Bioinformatics, ScRNA-seq data.

Introduction

Polydactylia, a common congenital limb abnormality, is characterized by the presence of extra fingers or toes. There are three types of polydactylia, including preaxial polydactyly (radial bone), central polydactyly (axial) and postaxial polydactyly (ulnar side). They are mostly inherited in an autosomal dominant manner, with varying penetrance, caused by defects in limb development before or after formation[1]. Currently, the only treatment method is surgical removal of redundant fingers or toes, which will not regenerate after the operation. The exact cause of polydactyly is still unclear. On the one hand, it is related to environmental factors, such as virus infections, drugs, radiation, etc., affecting the differentiation of embryonic limbs during early pregnancy. These factors may lead to finger differentiation disorders and limb deformities[2]. On the other hand, many patients show that their polydactyl disease-causing genes may be inherited from their parents[3].

With the further development of sequencing technology, some studies[4,5] have discovered important genes related to polydactyly, such as *HOX* genes, hedgehog pathway genes, FGFs, BMPs, and chondrogenic morphogenic proteins. These genes would lead to the occurrence of polydactyly by influencing limb development. Therefore, further research is needed to provide guidance for the identification of multiple-digit malformations as potential biomarkers and therapeutic targets.

Materials and Methods

Hypothesis

We speculate that in multiple-digital abnormalities, some transcription factors involved in the Sonic Hedgehog (SHH) signaling pathway will undergo heterogeneous changes. At the same time, these altered transcription factors will regulate the expression or dysregulation of a large number of target genes, thereby impacting cell functionality.

Retrieval and Process of Public Data

The unique molecular identified (UMI) count matrix derived from single-cell RNA-seq data from GSE158970 was retrieved. This matrix was then transformed into a Seurat object using the

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Seurat R package (version 4.0.4) (New York Genome Center, New York, NY, USA)[6]. Cells with a UMI count less than 500, detected genes less than 200, or over 30% of mitochondrial-derived UMI counts, were classified as low-quality cells and excluded. Additionally, genes detected in fewer than 5 cells were filtered out for further analyses.

ScRNA-Seq Data Preprocessing

Following quality control, the UMI count matrix underwent log normalization. Subsequently, the top 2,000 variable genes were utilized to generate potential anchors using the FindIntegrationAnchors function in Seurat. Then, the IntegrateData function was employed for data integration. In order to reduce the dimensionality of the scRNA-Seq dataset, principal component analysis (PCA) was conducted on an integrated data matrix. The top 50 PCs determined by the Elbowplot function in Seurat were applied for downstream analysis. The key cell clusters were identified using the FindClusters function in Seurat, with the resolution default set at 0.6. Visualization of these clusters was achieved through t-distributed stochastic neighbor embedding (tSNE) or Uniform Manifold Approximation and Projection (UMAP) plots. Cell types for each cluster were identified by detecting gene markers *via* the "FindMarkers" function in Seurat package (version 4.0.4), followed by annotation utilizing ScType tools (https://github.com/grisslab/scTypeR) and previously published marker genes[7].

Transcription Factor Regulatory Network Analysis

The SCENIC python workflow (version 0.11.2) (https://pypi.org/project/pyscenic/#files) was utilized to identify the modules of transcription factors (TFs) with default parameters[8]. A human TF gene list was obtained from the pySCENIC resources. Activated TFs were detected in the AUC matrix, and differentially activated TFs were chosen using the R package limma (https://www.rdocumentation.org/packages/limma/versions/3.28.14) [9]. The Regulon Specificity Score (RSS) was employed to identify cluster-specific regulons, especially in analyses involving numerous cell types with shared regulons[10]. The networks of TFs and their target genes within the modules were visualized using Cytoscape (version 3.9.1) (https://cytoscape.org/download.html).

Functional Enrichment Analysis

Functional categories of genes were categorized by identifying Gene Ontology (GO) terms and KEGG pathways using KOBAS 2.0 (http://bioinfo.org/kobas/download/)[11]. The enrichment of each term was defined using the hypergeometric test and Benjamini-Hochberg FDR controlling procedure.

Statistical Analysis

DEGs were identified using the FindMarkers/FindAllMarkers function within the Seurat package, utilizing a one-tailed Wilcoxon rank sum test with *p*-values corrected for multiple testing using the Bonferroni method. To identify Differentially Expressed Genes (DEGs), all genes were scrutinized for expression variances surpassing 0.5 on a natural logarithmic scale, with an adjusted *p*-value below 0.05. The Pheatmap package (available at: https://cran.r-project.org/web/packages/pheatmap/index.html) in R was used to perform the clustering based on Euclidean distance. Comparisons between the two groups were performed using the Student's *t*-test. The difference was considered significant when $p<0.05$.

Results

ScRNA-Seq Analysis of Extra-Digits from Polydactyly Patients Identified Distinct Cell Types

In order to explore more deeply the key regulatory factors of cell specificity in polydactyly development, we downloaded the data of GSE158970 polydactyly samples, including single-cell data of interdigital mesenchyme and epithelial cells from 4 polydactyly patients. First, after strict data quality control, we obtained transcriptional profile data of 11,806 single cells. We normalized the transcriptional expression matrix of these 11,806 single cells, performed principal component analysis, and selected the top 50 principal components for UMAP dimension reduction and visualization. After unbiased clustering analysis, we obtained 23 cell subgroups (clusters). Using the ScType software and combining it with the marker genes used in the original literature annotations, we identified 12 different cell types (Figure 1A-B, **Supplementary Figure 1**). At the same time, we also showed the expression of the top 3 marker genes and the significantly enriched functional pathways of marker genes ($|avg\_log_2FC|\geq 0.5$, $p\_adj\leq 0.05$) for each cell type (Figure 1C-D). We found that the marker genes and enriched functions corresponding to different cell types have

significant differences. For example, significant marker genes in melanocytes are mainly related to melanocyte differentiation, melanosome organization, and melanin biosynthesis, indicating that melanocytes may play an important role in melanin secretion and skin pigmentation. Next, we analyzed the proportions of each cell type in each sample (Figure 1E) and found that the main cell types include fibroblasts, keratinocytes, and endothelial cells.

Single-Cell Analysis Revealed Regulons that are Highly Correlated with Cell Function in Polydactyly

In order to investigate the regulation of regulons in different cell types of polydactyly, we calculated the activities of transcription factors in various cell populations. We respectively identified 14 and 4 regulons specifically activated in fibroblasts and keratinocytes (Figure 2A). Figure 2B respectively shows the regulatory elements associated with specificity for fibroblasts and keratinocytes. Regulons HOXD13 (+) and GLI2 (+) have higher AUC scores in fibroblasts, and the expression of these two transcription factors is also more specific in fibroblasts. Regulons HES2 (+) and GLIS1 (+) have higher AUC scores in corneocytes, and the expression of these two transcription factors is also relatively specific in corneocytes (Figure 2C). In order to further verify the regulatory function of these transcription factors, we constructed a regulatory network between the transcription factors and their target genes (Figure 2D). Among them, the number of target genes regulated by GLI2 in fibroblasts is abundant. The GO enrichment analysis demonstrates primarily enrichment in postsynaptic membrane assembly, dorsoventral patterning, and positive regulation of cell migration pathways (Figure 2E), indicating that the GLI2 transcription factor may be activated in the formation process of polydactyly. In addition, HOXD13 has also been widely reported to be closely related to the occurrence and development of polydactyly. Its mutation can cause limb abnormalities in humans and mice. HOXD13 is expressed in the interdigital mesenchyme and acts as an inhibitor of cartilage formation and cell differentiation. The lack of HOXD13 reduces the thickness and length of finger fusion, resulting in polydactyly due to the presence of extra, elongated fingers posterior to the axis. The target genes regulated by HES2 in corneocytes are mainly associated with transcriptional regulation (**Supplementary Figure 2**), GLIS1 regulates target genes mainly associated with proliferation and apoptotic pathways (Figure 2F), implying that they play a role in maintaining cellular function.

Transcription Factor Activities in Fibroblasts in Polydactyly

In order to further study the transcription factor activity in different subgroups of fibroblasts, we conducted a secondary clustering of fibroblasts, resulting in a total of five subgroups (Figure 3A), among them, C8 is identified as an onychofibroblas (matrix fibroblasts) based on the characteristic genes *RSPO4* and *MME* identified in the original literature (**Supplementary Figure 3A-B**). At the same time, we found that the expression of marker genes in each cell subgroup has obvious specificity (Figure 3B). Furthermore, these enriched functional pathways in cell subgroups also showed significant differences. For example, the C8 subgroup is mainly associated with endocardial cushion formation, neuronal development, and ameloblast differentiation (Figure 3C). Additionally, we calculated the specificity scores of regulons in each cell subgroup (Figure 3D). HOXD13, MSX2, and LHX2 were significantly activated in the C8 subgroup (Figure 3E), and their AUC activity and expression levels showed certain specificity in the C8 subgroup (Figure 3F). To further confirm the regulatory function of transcription factors, we constructed the regulatory network of HOXD13, MSX2, and LHX2 transcription factors and their target genes (Figure 3G). We found that the target genes of MSX2 and LHX2 were significantly enriched in pathways related to embryonic period specification or forelimb morphogenesis, and they were specifically highly expressed in the C8 subgroup (Figure 3H, **Supplementary Figure 3C-D**). These results imply that the C8 subpopulation of fibroblasts may be closely associated with the development of polydactyly and activate the related transcription factors to regulate the progression of polydactyly.

Transcription Factor Activities in Keratinocytes from Polydactyly Patients

In order to study the transcription factor activity of various cell subgroups in the stratum corneum cells more comprehensively, a secondary clustering of the stratum corneum cell population was conducted, resulting in 5 subgroups, including basal keratinocytes (Basal_kera), suprabasal keratinocytes (Suprabasal_kera), proliferating kerati-

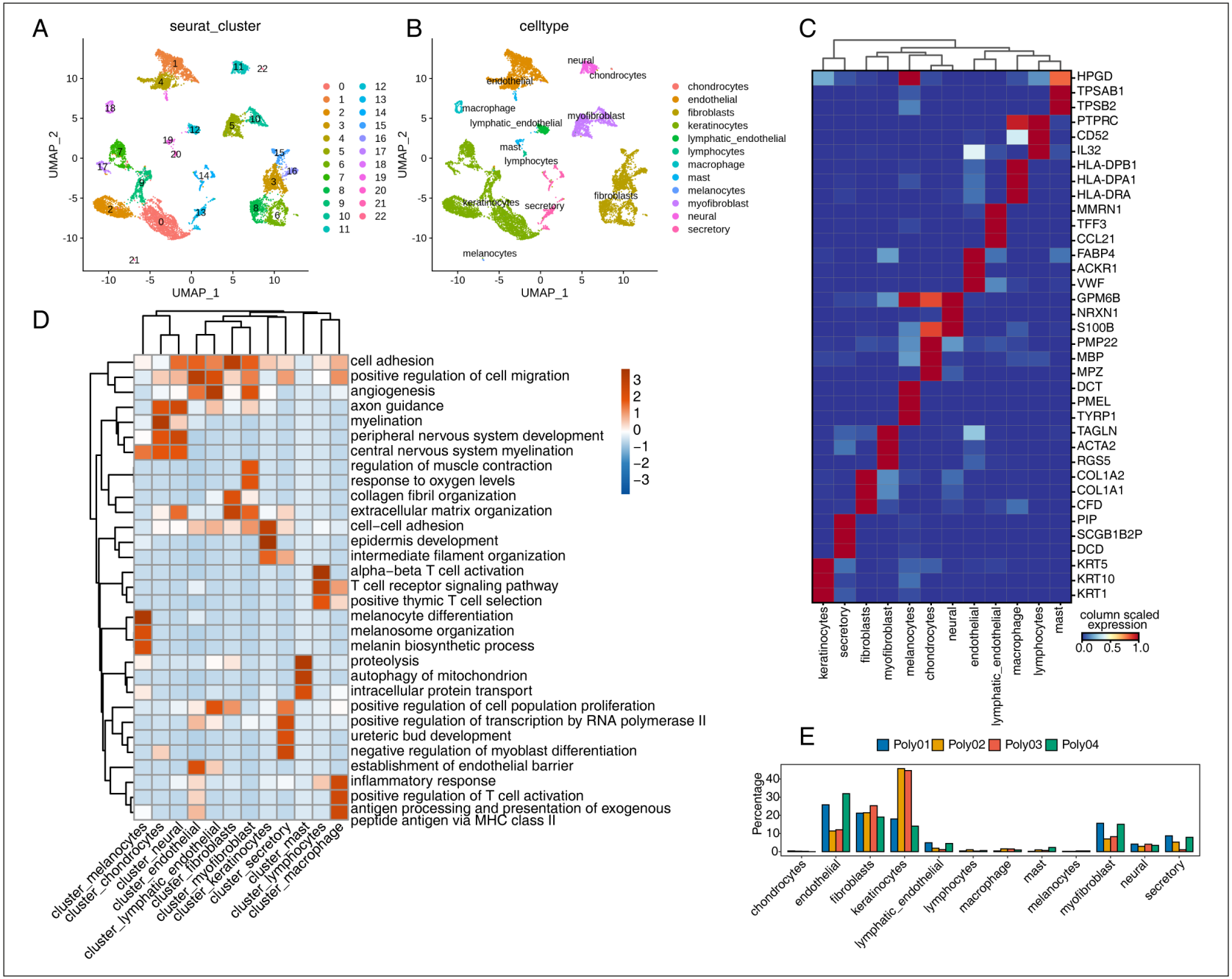

Figure 1. **A**, Based on Seurat, quality filtering, normalization, and dimension reduction analyses were performed on the single-cell data, resulting in the generation of a UMAP clustering plot. **B**, Cell type annotation was performed according to the markers provided in the original literature. **C**, The expression level variations of the top 3 marker genes in each cell type. **D**, GO functional pathway enrichment analysis was performed on significant marker genes in each cell type, the top 3 pathways for each cell type were chosen, and the q value (scaled by column) of these pathways was displayed using a heatmap to showcase the most enriched functional pathways for each cell type. **E**, The proportion of each cell type in the total population was compared among four samples.

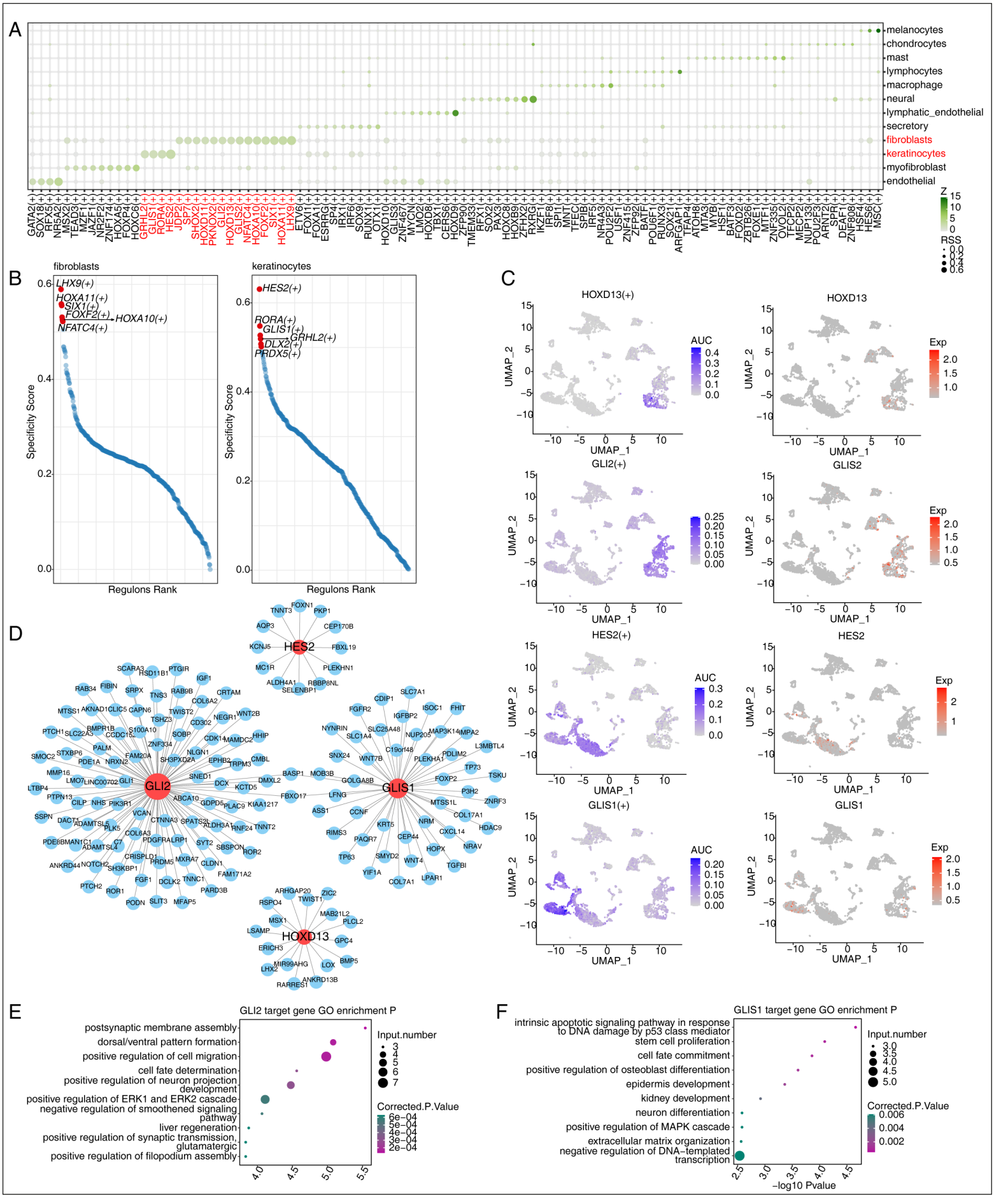

Figure 2. **A**, Regulons specificity scores in each cell population. **B**, Transcription factors specifically associated with fibroblast cells (left) and keratinocyte cells (right). **C**, The UMAP plot showcases the AUC activity levels and expression levels of transcription factors within cellular populations. **D**, Cytoscape displays the regulatory network composed of different active transcription factors and their target genes. **E**, *GLI2* transcription factor may be activated in the formation process of polydactyly. **F**, *GLIS1* regulates target genes mainly associated with proliferation and apoptotic pathways.

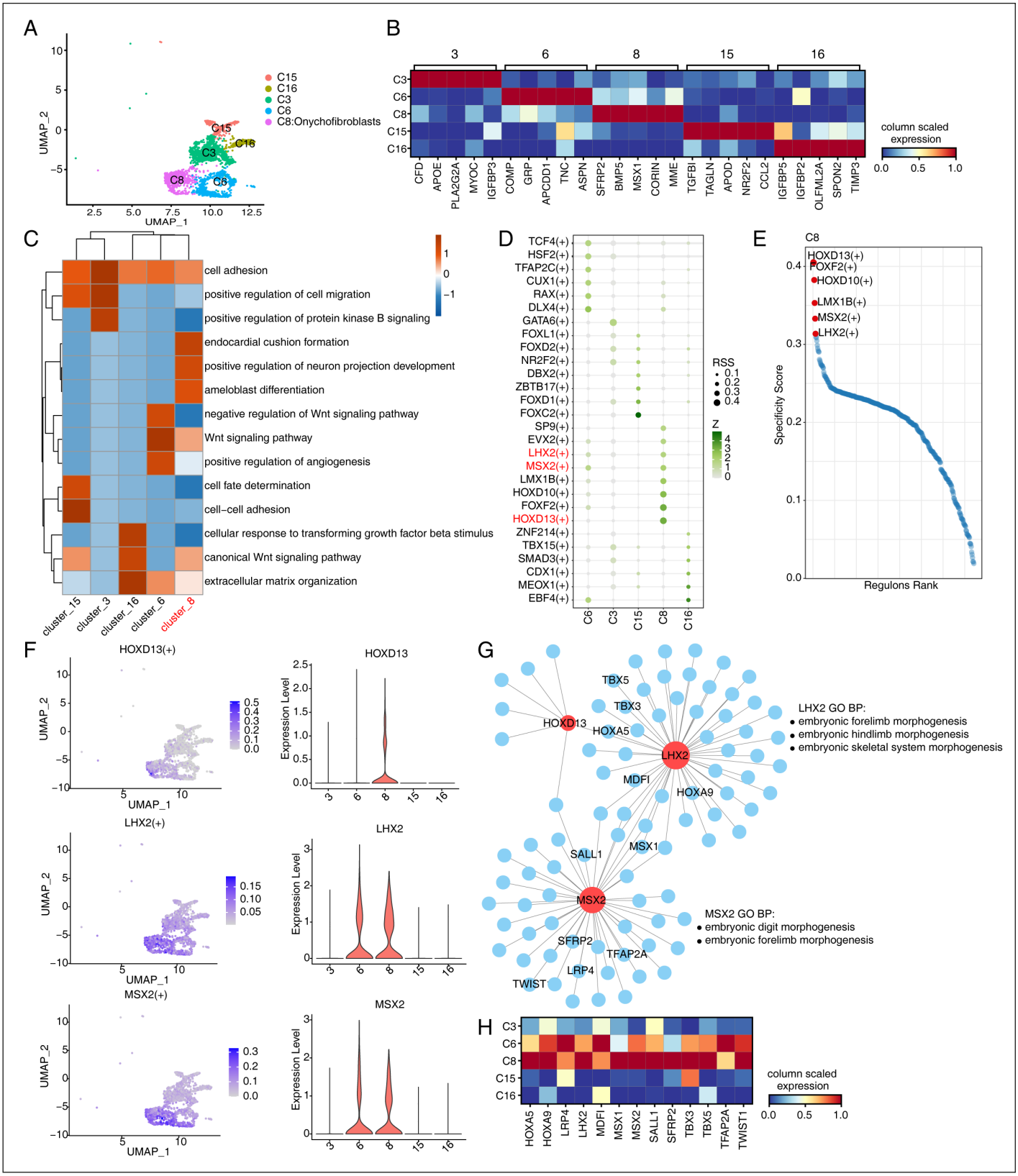

Figure 3. **A**, The extracted fibroblast cells were individually subjected to unsupervised clustering, and the UMAP plot displayed the obtained 5 cellular subclusters. **B**, The heat map displays the expression levels of the top 5 marker genes for each cell subtype. **C**, Perform GO functional pathway enrichment analysis on significantly marked genes in each cell subgroup. Select the top 3 pathways for each subgroup and display the q-value of these pathways using a heatmap (scaled by column). This will demonstrate the most enriched functional pathways for each subgroup. **D**, Calculate the specificity score of regulons in each cell subpopulation. **E**, Display the top six specificity-associated transcription factors in the C8 subpopulation. **F**, UMAP maps display the AUC activity levels of transcription factors, while Vlnplot showcases their expression levels. **G**, Cytoscape displays the regulatory network composed of transcription factors and their target genes.

nocytes (Proliferating_kera), and two types of nail keratinocytes (Nail_kera1 and Nail_kera2) (Figure 4A). It was found that the marker gene expressions and enriched functional pathways in these cell subgroups had both commonalities and specificities (Figure 4B-C). In order to further determine the specific transcription factors responsible for nail formation, the specificity scores of the regulons in each cell subgroup were calculated (Figure 4D). Among them, EMX2 and LEF1 were significantly activated in the Nail_kera1 subgroup, and both exhibited high AUC and expression levels in the Nail_kera1 subgroup. In addition, CREB3L2 and TGIF1 were specifically activated in the Nail_kera2 subgroup, and both exhibited high AUC and expression levels in the Nail_kera2 subgroup (Figure 4E). In order to further confirm the regulatory functions of the transcription factors, regulatory networks of EMX2, LEF1, CREB3L2, and TGIF1 with their target genes were constructed (Figure 4F). GO functional enrichment showed that the target genes of LEF1 were mainly enriched in signaling pathways such as extracellular matrix, cell proliferation, and angiogenesis, while the target genes of CREB3L2 were mainly enriched in processes such as apoptosis and cell adhesion; furthermore, these target genes were specifically highly expressed in the Nail_kera cell subgroups (Figure 4G, **Supplementary Figure 4**). Based on these results, it is inferred that the transcription factors in the Nail_kera keratinocyte subgroups may be closely related to nail formation and development.

Discussion

Transcription factors play an important role in multiple limb malformations; they exert their influence on gene expression in developmental gene regulatory networks by specifically interacting with cis-acting elements of eukaryotic genes[12]. Transcription factors are often the focus of research on polydactyly, especially zinc finger protein Kruppel family member *GLI1-3* has been reported to contribute to the occurrence and progression of polydactyl symptoms. This may be due to the alteration of the Sonic Hedgehog signaling pathway, resulting in abnormal limb development and leading to polydactyly[13-15]. Additionally, the GATA transcription factor serves as a crucial negative regulatory factor for ectopic SHH expression during limb development, particularly the loss of limb bud-specific *GATA6,* which may lead to ectopic expression of SHH and its target genes, thereby causing preaxial polydactyly[16]. Other transcription factors such as HOXD13, ETV4/5, Twist1, HAMD2, ALX4, which are related to SHH, have also been reported to be associated with the process of polydactyly[17-19]. Specifically, in regulating limb development, the SHH-GLI signaling axis is involved in the expression of many transcription factors responsible for polydactyly formation. Several hundred potential candidate genes regulated directly or indirectly by SHH and GLI transcription factors have been identified. However, we still know little about the expression patterns and related regulatory mechanisms of transcription factors that affect polydactyly in different types of cells.

The single-cell RNA sequencing (scRNA-seq) technology provides a favorable tool for studying the heterogeneity and dynamic regulation of multiple abnormal cells. Cells are the basic units that makeup tissues and organs. Previous sequencing technologies have focused on studying cells collectively, reflecting the average level of cell clusters. However, the information and expression levels vary greatly between cells, making it impossible to objectively reflect the status of disease occurrence and development in individual cells. Single-cell RNA sequencing (scRNA-seq) enables amplification and sequencing of the entire transcriptome at the single-cell level. The principle involves amplifying the isolated individual cell's total transcriptome RNA, followed by high-throughput sequencing. This technology enables the detection of gene expression states at the single-cell level, providing an accurate depiction of the heterogeneity among cells[20,21], revealing the diversity of cells in the organs[22] and constructing intercellular networks between different cell populations[23]. Therefore, single-cell transcriptome sequencing has been widely used to detect gene expression profiles of different types of cells in reproductive, developmental, and disease processes, thereby revealing the molecular mechanisms of different cells' roles and functions in these processes.

Significant findings have been made regarding the cell-specific gene expression profiles during the development and progression of diseases through single-cell RNA sequencing (scRNA-seq). However, there have been no reports on the research of polydactyly on a single-cell level, except for one report[24] that used polydactyly samples to study the single-cell transcriptome profile of human nails. In that report, Kim et al[24] used four polydactyly patient samples for single-cell sequencing, and analyzed the characteristics of the specific mesenchymal matrix fibroblasts and epithelial cell clusters in

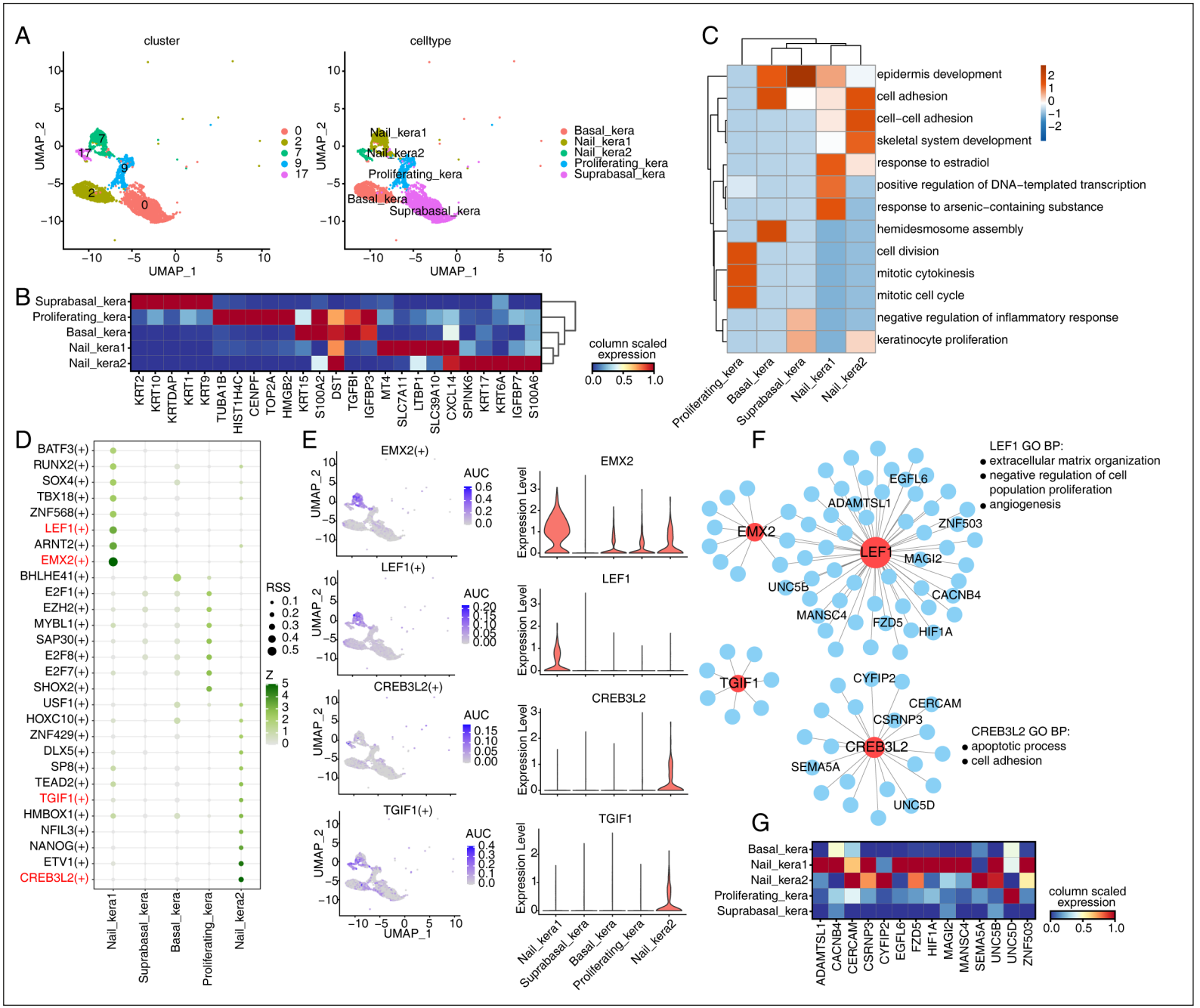

Figure 4. **A**, keratinocyte cells were extracted separately for unsupervised clustering, UMAP graph displays the obtained 5 cell subgroups. **B**, The heatmap displayed the expression levels of the top 5 marker genes for each cell subpopulation. **C**, GO functional pathway enrichment analysis was performed on significant marker genes from each cell subpopulation. Top 3 pathways were chosen for each subpopulation and display the q-values of these pathways in a heatmap (scaled by column). This will demonstrate the most enriched functional pathways for each subpopulation. **D**, Specificity score of regulons in each cell subpopulation was calculated. **E**, UMAP and Vlnplot graphs respectively displayed the AUC activity levels and expression levels of various transcription factors in different subgroups. **F**, Cytoscape displayed a regulatory network composed of transcription factors and their target genes.

human nails as *RSPO4* and *SPINK6*, respectively. They also found that *RSPO4* matrix fibroblasts interact with LGR6 nail matrix epithelial cells, promoting nail formation and growth through the WNT/β-catenin signaling pathway[24].

In our study, ScRNA-seq analysis of extra-digits from polydactyly patients identified distinct cell types. Our division and annotation of cell groups are generally consistent with the original data. In the current study, analysis of the transcriptional profile data of 11,806 single cells revealed that regulons that are highly correlated with cell function in polydactyly, the function of HOX family members and GLI2 transcription factors such as HOXD13, MSX2, LHX2, EMX2, LEF1, and CREB3L2 in the process of polydactyly in fibroblast cells, and roles of HES2 and GLIS1 in the process of keratinocyte formation and development. Additionally, we compared the marker genes and enriched functional pathways of each cell population. For the first time, a detailed and in-depth analysis of regulons was conducted using multiple congenital single-cell data. We found that transcription factors in different cell populations exhibit a high degree of heterogeneity dominated by fibroblasts and keratinocytes. Through analyzing the regulons in fibroblasts, we discovered a significant presence of transcription factors related to polydactyly in a subgroup of fibroblasts, with particular emphasis on HOXD13, MSX2, and LHX2. Additionally, through functional enrichment analysis of target genes regulated by these transcription factors, we found that they may be closely related to limb morphology, providing new insights into the regulatory mechanisms of transcription factors in polydactyly. Through the analysis of regulons in cornified cells, we discovered the existence of two subgroups of cornified cells, in which some transcription factors they contain may be vital in nail formation and development.

To the best of our knowledge, the current study is the first attempt to carry out bioinformatics analysis of the data on polydactyly so as to provide novel transcription factors and genes for the diagnosis and treatment of polydactyly. The broader implications of our results for understanding the pathogenesis of polydactyly are as follows: 1) Studying the molecular mechanisms underlying polydactyly can provide insights into normal limb development. By identifying the specific transcription factors and signaling pathways involved, researchers can gain a deeper understanding of the regulatory networks that control limb patterning and formation. 2) Although polydactyly can have both genetic and environmental factors, understanding the genetic basis of polydactyly, including the involvement of specific transcription factors, can help in assessing the risk of recurrence in families affected by polydactyly and provide information for making informed reproductive decisions. 3) Identifying the key transcription factors and molecular pathways involved in polydactyly can potentially open doors for developing targeted therapies. Targeting specific transcription factors or modulating their activity may offer potential treatment strategies to correct or prevent polydactyly. This could involve gene editing techniques, small molecule inhibitors, or other interventions that aim to normalize gene expression patterns during limb development. 4) Understanding the molecular mechanisms behind polydactyly can also contribute to advancements in regenerative medicine. By elucidating the signals and factors necessary for limb development, researchers may gain insights into techniques for inducing or enhancing limb regeneration in cases of amputation or other limb defects.

In the meanwhile, aside from genetic variations associated with polydactyly, the independent contribution of other factors beyond these known genetic variants should also be considered. For example, environmental factors such as maternal exposure to certain substances, gestational conditions, and lifestyle factors could potentially influence polydactyly. We wish to have incorporated these factors into our analysis models to determine their association with polydactyly while accounting for their potentially confounding effects, but it was not possible due to the nature of the data this bioinformatics study is based on. It is necessary to obtain a more comprehensive understanding of the development of polydactyly and to ensure that our findings are not solely attributed to genetic or environmental factors alone.

Conclusions

Significant presence of transcription factors, especially HOXD13, MSX2, and LHX2, may be strongly related to the development of polydactyly.

Conflict of Interest

The authors declare no conflicts of interest regarding this manuscript.

Ethics Approval
Ethical approval is unnecessary as this study involves analyzing pre-existing publicly accessible data and does not entail any experimentation on living organisms, cells, or tissues.

Informed Consent
Informed Consent is not required since this analysis utilized publicly available data and did not involve any human subjects.

Availability of Data and Materials
Datasets can be accessed from the corresponding author upon request.

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Authors' Contributions
DF extracted and analyzed the data and wrote the manuscript. AM designed the study, analyzed data, and revised the manuscript. All authors reviewed and approved the last version of the manuscript.

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