Characteristic analysis of skin keratinocytes in patients with type 2 diabetes based on the single-cell levels

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Abstract

Background: Keratinocytes play an important role in wound healing; however, less is known about skin keratinocytes in patients with type 2 diabetes mellitus (T2DM). Therefore, this study aimed to search for the transcriptional characteristics of keratinocytes at the single-cell level from T2DM patients, and to provide experimental data for identifying the pathological mechanisms of keratinocytes under pathological conditions.

Methods: We performed single-cell RNA sequencing on the skin tissue from two T2DM patients and one patient without diabetes-induced trauma using the BD Rhapsody™ Single-Cell Analysis System. With the help of bioinformatics R-based single-cell analysis software, we analyzed the results of single-cell sequencing to identify the single-cell subsets and transcriptional characteristics of keratinocytes at the single-cell level, including Kyoto Encyclopedia of Genes and Genomes and Gene Ontology analyzes.

Results: In this study, we found specific highly expressed signature keratinocyte-related genes. We analyzed the transcriptome of keratinocytes from experimental and control groups and screened a total of 356 differential genes, which were subject to bioinformatics analysis. Enriched pathways included oxidative phosphorylation, antigen processing and presentation, prion and Huntingtons' diseases, bacterial invasion of epithelial cells, thermogenesis, vasopressin-regulated water reabsorption, and protein processing in the endoplasmic reticulum.

Conclusions: This study revealed the characteristics of keratinocytes at the single-cell level and screened a group of differentially expressed genes related to T2DM-associated keratinocytes, oxidative phosphorylation, cytokine receptor interactions, prion diseases, and other signaling pathways.

Keywords: Diabetes mellitus, type 2; Gene Ontology; Keratinocytes; Prion diseases; Sequence analysis, RNA; Single-cell analysis; Transcriptome; Wound healing

Introduction

Wound healing is a complex process that includes a series of pathophysiological processes, including inflammatory responses, epidermal regeneration, proliferation and differentiation of various cells, and tissue remodeling.[1] The epidermis forms the protective structure of the outer layer of the human body. Under physiological conditions, the epidermis is constantly undergoing self-renewal after being stimulated by exogenous and endogenous injuries. The main participants in this process are the keratinocytes that are located in the basal layer of the epidermis.[2] As the wound forms, the process of epidermal regeneration is also completed by keratinocytes through the processes of proliferation, differentiation, and migration. Therefore, any endogenous or exogenous adverse factors that could interfere with the regulation of wound healing will affect the wound healing process.[3] In patients with type 2 diabetes mellitus (T2DM), especially in those whose blood glucose is not well controlled, delayed union or even non-union in wound healing is common. However, the mechanism behind this impaired wound healing still needs further clarification, including the pathological changes of keratinocytes that are in a high glucose state.

Compared with bulk RNA sequencing (RNA-seq), which only provides an average expression for millions of cells, single-cell RNA sequencing (scRNA-seq) allows concurrent analysis of thousands of cell transcriptomes at the single-cell level, thereby allowing for the characterization of novel cell subsets.[4] Therefore, scRNA-seq can reliably identify even closely related cell populations, reveal changes that make each cell type unique, clarify the heterogeneity of gene expression patterns in peripheral blood cell populations, and be applied in the diagnostic and prognostic evaluation of clinical diseases.[5,6]

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However, current skin tissue studies are still insufficient to fully elucidate the mechanisms of wound healing, at the single-cell level. Therefore, in this study, we collected skin tissues from two patients with T2DM and one patient without diabetes and performed scRNA-seq of the tissues. This study aimed to investigate the characteristics of keratinocytes at the single-cell level in patients with diabetes and to provide a scientific basis for the care and treatment of clinical wound healing.

Methods

Ethics approval

The study was conducted according to the *Declaration of Helsinki*. The Ethics Committee of Integrated Traditional Chinese and Western Medicine Hospital, Southern Medical University, approved the protocol in the form of case reports. Written informed consent was obtained from each patient.

Human samples

Samples were collected from two patients with T2DM (47 years and 58 years, male, abdominal surgery) and one trauma patient without diabetes (52 years, male, right upper extremity trauma) as the control. Skin tissue was obtained from resected tissue at the time of patient's surgery. Primary tissue preparation was performed according to the previous studies. Briefly, the collected tissues were minced and digested into a single-cell suspension, and single cells were prepared in the differentiation medium. After centrifugation, the cells were resuspended in Roswell Park Memorial Institute medium with 10% fetal bovine serum (RPMI-1640 + 10% FBS) and processed to construct single-cell next-generation sequencing libraries.

Single-cell RNA sequencing

Cells from each sample were labeled with sample tags (BD™ Human Single-Cell Multiplexing Kit, BD Biosciences, San Jose, CA, USA) following the manufacturer's protocol. The viability of cells in all samples assessed was >80%. Labeled samples were pooled equally in cold BD sample buffer with approximately 30,000 cells per sample. Single cells were isolated using Single-Cell Capture and complementary DNA (cDNA) Synthesis with the BD Rhapsody Express Single-Cell Analysis System, and cDNA libraries were prepared using the BD Rhapsody™ Whole Transcriptome Analysis Amplification Kit. The final library was quantified using Qubit fluorometry with Qubit-dsDNA-HS Kit (ThermoFisher, Wilmington, DE, USA). Libraries were sequenced in paired-end mode on a NovaSeq 6000 in the lab of Novogene Biotech Co., Ltd. (Beijing, China).

Processing and analysis of single-cell RNA-seq data

Raw data read from scRNA-seq were processed using R 3.6.2, and gene expression data analysis were performed using the R/Seurat package. For the quality control step, low-quality cells, empty droplets, and multiplexed captures were first filtered out based on the distribution of unique genes detected in each cell in each sample. Cells with <300 genes and cells with >6000 genes were excluded. The cellular distribution of the expression-based mitochondrial gene fraction was also plotted, and cells with a mitochondrial gene fraction >30% were discarded to eliminate dying cells or low mass cells with extensive mitochondrial contamination. Subsequently, t-distributed Stochastic Neighbor Embedding (tSNE) was used for the two-dimensional representation of data structures. After clustering, the cluster biomarkers of each group were searched using the findmarkers function and findallmarkers function within the Seurat package; thus, we were able to define the marker genes of clustering by their differences in expression.

We performed differential expression analysis for the experimental and control groups and identified the sets of differentially expressed genes in R using DESeq2 package with threshold set at $P < 0.05$ and absolute value of fold change >1.5. The identified differentially expressed genes were analyzed according to Gene Ontology (GO) functional categories and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment pathways.

Results

Single-cell analysis reveals heterogeneity in skin tissue

We performed a routine pathological examination of the collected skin tissue, and the layers of the skin tissue were visible [Figure 1A]. Then, we performed scRNA-seq on skin and subcutaneous tissue, and after performing the quality control, approximately 21,819 cells met quality control metrics and were analyzed [Figure 1B–D]. Unsupervised clustering using the Seurat package identified eight clusters of cells (clust 0–7; Figure 1E). Using differentially expressed gene signatures, we attributed the clustering to their putative identity. Cluster C0 cells were classified as endothelial ($n = 6101$; 57.66%), and they expressed *ACKR1, CD93, ALCRL, FLT1, S1PR1, TM4SF1, PCAT19, KDR, MMRN1, CLDN5, STC1, PODXL, SOX18, CD200*, and *SELE*. Cluster C1 cells were classified as fibroblasts ($n = 1792$; 16.94%) and C2 as smooth muscle cells ($n = 1134$; 10.72%). Cluster C3 cells ($n = 520$; 4.91%) were classified as keratinocytes based on expression of *SFN, AQP3, LGALS7, KRT1, DSC3, TRIM29, DMKN, KRT10, GJB3, S100A14, KRT16, KRT5, PKP3*, and *KRT14*. Cluster C4 cells were classified as another fibroblast cluster ($n = 349$; 3.30%), and they expressed *FN1, DPT, TNFAIP6, LUM, DCN, COL8A1, ANGPTL2, EFEMP1, BGN, SCARA5, ASPN, COL14A1, IGFBP6*, and *MMP14*. Cluster C5 cells ($n = 520$; 4.91%) were classified as dendritic cells ($n = 291$; 2.75%) based on expression of *LYZ, MS4A6A, HLA-DRA, HLA-DPB1, HLA-DPA1, FCER1G, CD74, HLA-DRB1*, and *CD86*. Two other distinct cell clusters were C6 and C7 ($n = 286$; 2.7% and $n = 108$; 1.02%, respectively). Cluster C6 cells were enriched and used for mast cells markers *CPA3, KIT, TPSAB1, TPSB2, VWA5A, CMA1, RAB27B, IL1RL1, ADORA3, FCER1A*, and *GATA2*, while cluster C7 cells were used for T-cells markers *CD40LG, CD3D, ICOS, CD3G, GZMB, KLRB1, CD2, NKG7, TRBC2, CD3E, GZMA*, and *CCL5*.

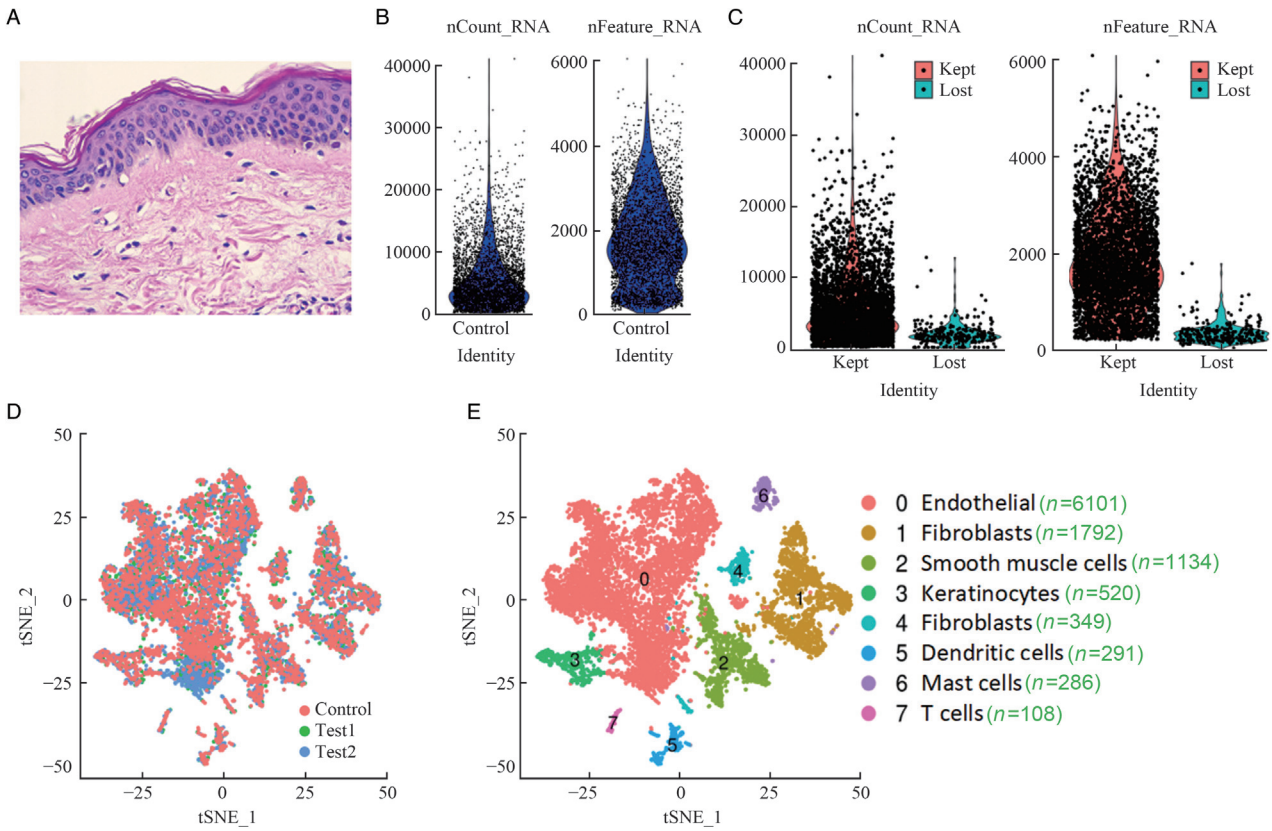

Figure 1: Single-cell transcriptomic landscape of keratinocytes. (A) Pathological section of skin with Hematoxylin and Eosin stain, 10 × original magnification. (B) The count and feature of unprocessed data (control). (C) The retained and lost count and features of the sample. (D) The tSNE map of the three samples (tests1 and 2 are T2DM). (E) Identification and annotation of cell subsets. tSNE: t-distributed Stochastic Neighbor Embedding; T2DM: Type 2 diabetes mellitus.

Recognition and identification of keratinocytes

After annotation of cell population, we identified cluster 3 ($n = 520$) as keratinocytes. We found that the specific expression of signature genes of keratinocytes supported the recognition of this group as keratinocytes, with specific highly expressed *SFN, LYPD3, S100A8, KRT1, KRT10, KRT6A, KRT5,* and *KRT16* [Figure 2]. *KRT* belongs to the keratin family, a family of fibrous structural proteins known as sclerosis. Keratin is a key structural material that forms the outer layers of scales, hair, feathers, horns, feet, hooves, calluses, and vertebrate skin. Keratin can also protect the epithelial cells from damage or stress.

Transcriptional characteristics of keratinocytes from T2DM patients

We analyzed the transcriptome of keratinocytes from the T2DM and the control group and screened a total of 356 differential genes at $P < 0.01$, including *LUCAT1, MAL2, MXD1, PKP1, JUP, MARCH5, MARCH7, PTEN, AQP9, PIK0R3, PHKA2, PC, IL3, ADH4*, etc [Figure 3A]. Gene set enrichment analysis revealed differential gene enrichment of signaling pathways including oxidative phosphorylation, cytokine–cytokine receptor interactions, prion disease, Huntington's disease, antigen processing and presentation, thermogenesis, tryptophan metabolism, retinol metabolism, amyotrophic lateral sclerosis, fatty acid degradation, tyrosine metabolism, bacterial invasion of epithelial cells, and other processes [Figure 3B]. Among them, for prion disease signaling pathway, normalized enrichment score (NES) = 1.585, $P = 0.0029$; for oxidative phosphorylation signaling pathway, NES = 1.733, $P < 0.0001$; cytokine–cytokine receptor interactions signaling pathway, NES = 1.598, $P = 0.0030$ [Figure 3C–E]. The Gene Ontology term enrichment analysis revealed that the specific highly expressed genes in keratinocytes were enriched in biological process terms related to the regulation of T cell activation, leukocyte cell–cell adhesion, regulation of lymphocyte activation, lymphocyte differentiation, positive regulation of cytokine production, regulation of hemopoiesis, cellular responses to tumor necrosis factor (TNF), negative regulation of interleukin-2 production, cytokine secretion, the cellular component terms related to the external side of the plasma membrane, focal adhesion, cell-substrate adherens junction, invadopodium, condensed chromosome, and molecular functions related to actin binding.

Discussion

Patients with T2DM have an increased risk of skin infection and poor wound healing. Impaired keratinocyte function is one of the major factors for impaired wound healing in patients with diabetes.[7] In the pathological changes of abnormal cellular immune function and tissue inflammation, the keratinocyte defense response plays an important role in these pathological changes and is usually called a defensive sentinel.[8]

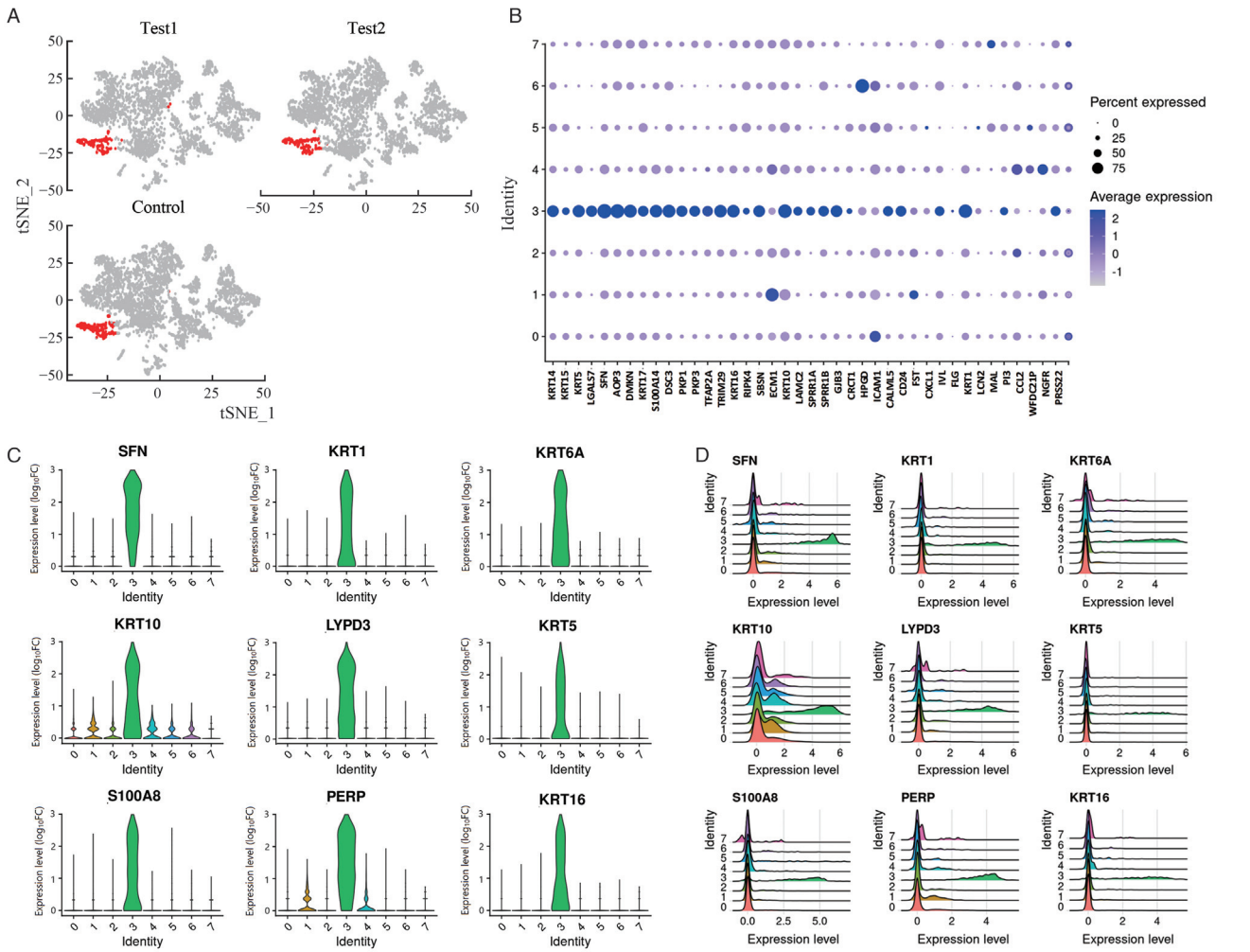

Figure 2: Identification and analysis of keratinocytes from T2DM patients. (A) The tSNE map of keratinocytes shows that the proportion of keratinocytes cells is more dominant. (B) Keratinocytes clusters were identified by specific markers expression. (C) Violin plots show the presence of key markers for keratinocytes. (D) Ridge plots show the presence of key markers for keratinocytes. tSNE: t-distributed Stochastic Neighbor Embedding; T2DM: Type 2 diabetes mellitus.

In this study, we collected skin tissue from T2DM patients, and then performed scRNA-seq based on BD Rhapsody. Cells were divided into eight clusters, and seven kinds of cell types were identified, such as endothelial, fibroblasts, smooth muscle, keratinocytes, dendritic, mast, and T-cells. We first characterized keratinocytes and were able to clearly discover the high expression of the *KRT* family of proteins in agreement with keratinocyte function. However, the number of keratinocytes screened in this study was not large; on the one hand, relatively more subcutaneous tissue was attached to the tissue, on the other hand, cells that might be close to the skin surface were in a keratinized state and could easily be treated as low-quality cells that had lost their activity, and thus, were filtered out during the data preprocessing phase. At the same time, we found that *S100A8* was highly expressed in keratinocytes. *S100A8* and its binding partner *S100A9* are members of a non-ubiquitous multigene family of cytoplasmic Ca^{2+}-binding proteins.[9,10] Because of their damage-associated molecular pattern, their differential expression in chronic inflammatory diseases, and their association with cancer, these proteins have received much attention over the past years.[9] The expression of *S100A8* is specifically derived from activated phagocytes.[11] It has been previously documented that *S100A8/A9* is found in differentiating supra-basal wound keratinocytes,[12] particularly during the first 12 to 24 h after injury, and was found to gradually return to baseline expression within two weeks after injury.[13] Therefore, during the wound healing period, the *S100A8/A9* immunoreactivity map increased rapidly and infiltrated into the collected leukocytes followed by continuous *S100A8/A9* expression in the keratinocytes of the wound, representing *de novo* synthesis of *S100A8/A9*.[14,15]

Compared with non-diabetic samples, we screened high and low expression genes of keratinocytes from T2DM. Kyoto Encyclopedia of Genes and Genomes pathway analysis according to differential genes identified about 20 differentially enriched signaling pathways, of which, the top ranked ones were oxidative phosphorylation, cytokine–cytokine receptor interactions, and prion disease. Oxidative phosphorylation is an efficient way of producing large amounts of adenosine triphosphate (ATP), thus producing chemical ATP gradients in the process.[16] The most important part of this process is the electron transfer

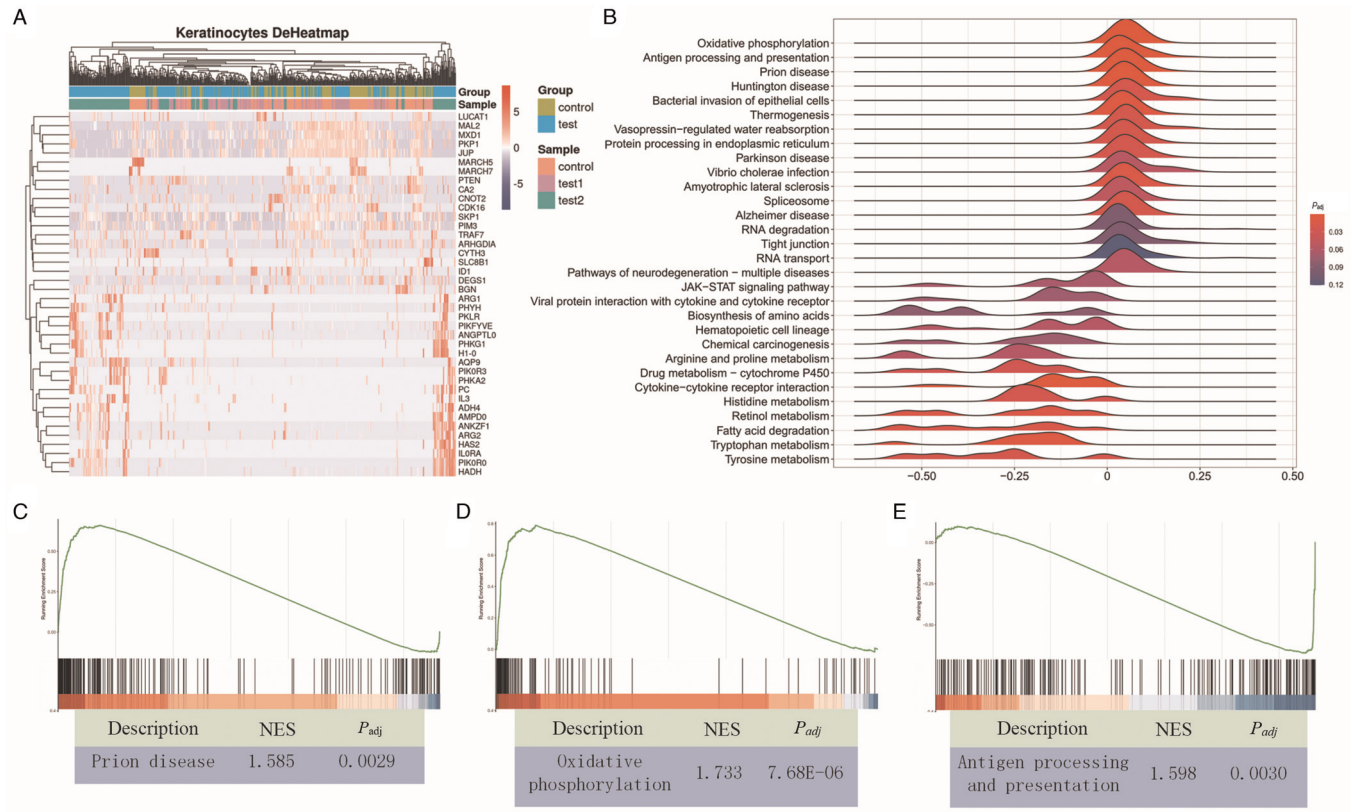

Figure 3: Characteristic analysis of keratinocytes. (A) Heat map of differential genes, (B) KEGG and GO pathway enrichment of differential genes. (C–E) The top three enriched pathways. GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; NES: normalized enrichment score.

chain, which produces more ATP than any other step in cellular respiration. Therefore, the abnormal signaling pathway may suggest that the energy metabolism pathways and methods of keratinocytes in T2DM patients may be abnormal. Another enrichment signal pathway consists of cytokine–receptor interactions, a common, but not highly specific signaling pathway, especially in inflammatory and immune-related diseases.[17,18] Cytokines participate in innate and adaptive inflammation, host defenses, cell growth, differentiation, cell death, angiogenesis, and key intercellular modulators and also cause mobilization of cells that aim to restore homeostasis in the development and repair process.[19] Keratinocytes express and secrete a broad range of cytokines that can affect and amplify inflammatory responses, induce keratinocyte proliferation, and promote the migration of leukocytes into the skin.[20,21] It is reported that keratinocytes constitutively produce interleukin (IL)-1α and -1β, which bind to the same receptor complex and have similar biological activities.[22,23] IL-1 released by keratinocytes can trigger a rapid immune response, leading to the expression and release of other cytokines such as IL-6, IL-8, and tumor necrosis factor (TNF), leading to Th2 cytokine-induced activation of the IL-33/ST2 axis, which is involved in the progression of several skin diseases.[24,25]

The limitations of this study are the small number of subjects, and in addition, the enrolled diabetic patients had relatively good glycemic control and did not fully represent the abnormal glucose metabolism found in pathological conditions. Moreover, the control group was accompanied by a certain skin inflammatory response, which could have affected the objectivity of the experimental data results to some extent. Finally, although the present study analyzed the characteristics of keratinocytes at the single-cell level, cellular experiments and animal experiments would be more desirable if the molecular and biological alterations of keratinocytes in diabetic patients are to be elucidated.

In conclusion, single-cell sequencing from the skin samples of patients with T2DM was performed. Characteristics of keratinocyte at the single-cell levels were revealed, and a group of differentially expressed genes of keratinocyte related to T2DM, which were enriched in oxidative phosphorylation, cytokine receptor interaction, prion disease, and other signaling pathways were screened. Thus, our study provides valuable experimental data concerning the impairment of wound healing caused by diabetes and helps provide clues for the future studies on the molecular mechanisms of would healing.

Conflicts of interest

None.

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