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Review

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Review

Decoding the molecular landscape of keloids: new insights from single-cell transcriptomics

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Abstract

Keloids are a fibrotic disease caused by an excessive accumulation of extracellular matrix in the dermis; they have neoplasia-like properties of aggressive growth and high posttreatment recurrence rates. Therefore, it is imperative to gain additional insight into the pathobiology of keloid formation. Single-cell RNA sequencing (scRNA-seq) technology has brought data-driven innovation to understanding the pathogenesis of keloids by breaking the limitations of traditional sequencing technologies to resolve cell composition and to distinguish functional cell subtypes at an unprecedented resolution. The present review aims to cover the application of scRNA-seq technology in keloids and its exploratory findings, including the depiction of the cellular landscape of keloids, fibroblast heterogeneity, the lineage development of Schwann cells and the mesenchymal-activation phenomenon of endothelial cells. Furthermore, scRNA-seq records the transcriptional profiles of fibroblasts and immune cells in a more refined manner, and this gene expression information provides excellent material for inferring intercellular communication networks and lays an important theoretical foundation for future studies.

Key words: Keloid, scRNA-seq, Fibroblast, Schwann cell, Immune cell, Transcriptomics

Highlights

- This paper first reviews the application of single-cell RNA sequencing technology in keloids and its exploratory findings.
- Single-cell sequencing reveals the heterogeneity of fibroblasts and the developmental trajectory between different functional groups and resolves the comprehensive landscape of the immune microenvironment of keloids.
- The transcriptional profiling data obtained by single-cell sequencing will provide an adequate theoretical basis for subsequent experimental studies.

Background

Keloid scarring evolves from dysfunctional wound healing after skin injury and pathologically manifests as an excessive accumulation of extracellular matrix in the dermis. This refractory skin disease is characterized by neoplastic-like properties, namely, aggressive growth, difficulty in spontaneous regression and a high posttreatment recurrence rate [1,2]. Surgical excision of the lesions combined with radiotherapy or pharmacological treatment (e.g. triamcinolone, 5-fluorouracil, bleomycin, etc.) has been recommended as the

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first-line treatment for keloids, and these standard treatments have not been revolutionized in the past decade [3,4]. Despite the gradual introduction of encouraging therapies, such as cryotherapy, into the routine treatment of keloids, long-term clinical outcomes remain poor due to the high post-treatment relapse rate [5,6]. A thorough understanding of the molecular mechanisms of keloid scarring may provide ideas and a research basis for improving existing treatment options and developing systemic or targeted therapies.

The application of sequencing technology to the study of keloids is a milestone in gaining insight into their molecular status. It allows the bulk identification of multiple differentially expressed genes in keloids, involving a variety of biological processes such as cell proliferation, apoptosis, extracellular matrix deposition, angiogenesis and inflammatory response. A combination of bioinformatics techniques and machine learning algorithms has facilitated the investigation of immune cell infiltration and the discovery of multiple biomolecules in keloids; for instance, LGR5 and DKK1 have been identified as keloid-associated immune signaling molecules [7]. Moreover, non-coding RNA sequencing of keloid (including microRNAs (miRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs)] have identified multiple differentially expressed miRNAs (e.g. miR-21 and miR-205-5p), lncRNAs (e.g. lncRNA DLEU2 and lncRNA H19) and circRNAs (e.g. hsa_circ_0057452 and hsa_circ_0007482) [8–10]. Zhong *et al*. detected 264 significantly altered miRNAs in keloid and revealed that these miRNAs were associated with mitogen-activated protein kinase (MAPK) and hypoxia inducible factor-1 (HIF-1) signaling pathway activity based on enrichment analysis [11]. The identified miRNAs and lncRNAs constructed multiple pairs of competing endogenous RNA networks involved in keloid formation by regulating gene expression, transcriptional modifications and histone modifications [12,13]. In addition, a large number of statistically significant differentially methylated cytosine phosphodiester bond guanines (CpGs) between keloids and normal tissues were identified and mapped to 152 genes, systematically depicting the methylation landscape of the keloid genome [14]. Although high-throughput sequencing has provided information on gene expression levels for the understanding of keloids, traditional sequencing techniques treat keloids as a whole, ignoring the unique transcriptional profiles of certain cells and obscuring the discovery of rare cell subpopulations [15]. New biological questions, e.g. the distribution of cell types in keloids, the stochasticity of gene expression and intercellular communication networks, remain unanswered and are major obstacles to achieving precision medicine. There is a pressing demand to unmask the cellular composition and individual cell-specific transcriptional profiles of keloids at a higher resolution. Fortunately, the development of single-cell sequencing technology has provided a powerful tool to capture the intercellular heterogeneity of keloids.

Review

Single-cell RNA sequencing

Emerging single-cell sequencing technologies include high-throughput sequencing of the genome, transcriptome and epigenome at the individual cell level, with the broadest application being single-cell RNA sequencing (scRNA-seq). scRNA-seq technology allows the differentiation of diverse cell types in complex population assemblies, breaking the bottleneck of traditional sequencing and providing a powerful approach to learn about the processes underlying diseases [16,17]. The implementation of scRNA-seq technology generally involves individual cell isolation, reverse transcription, complementary DNA (cDNA) amplification, library preparation and data analysis, and its successful execution cannot be achieved without advances in bioinformatics, statistics and genetics [18–20].

Collected fresh tissues require digestion and dissociation into single-cell suspensions, followed by washing, filtration, counting and a cell viability assessment (Figure 1). The obtained RNA is reverse transcribed, and cDNA products are collected and amplified for sequencing [21]. Advancements in separation techniques such as flow cytometry sorting, laser capture microdissection and microfluidics during single-cell collection have fueled higher throughput for sequencing. In reverse transcription, the introduction of a unique molecular identifier, which is the addition of barcodes of 4–10 bp in length to the 5′ or 3′ end of each cDNA, can substantially eliminate gene length differences and reduce background noise caused by amplification, allowing more accurate quantification of the initial amount of transcripts in single cells [22,23]. The computational difficulties caused by the massive amount of gene information have been addressed by scientists through the projection of data into the 2D space and feature selection. Widely applied dimensionality reduction methods include principal component analysis, t-distribution stochastic neighbor embedding and diffusion maps, which reduce the dimensionality of the data and maximize the retention of certain characteristic information of the original data [24,25].

The combination of cutting-edge algorithms and mature visualization methods, such as clustering and pseudotime analyses, has brought tremendous convenience to the exploration of sequencing data [26,27]. The clustering algorithm classifies a large number of cells into different subgroups based on their gene expression, which facilitates the discovery of gene expression that is overlooked in population-based RNA sequencing but is meaningful in some cells. Pseudotime analysis constructs developmental trajectories of cells to infer cellular changes over time, which is biologically significant for studying the transformation of immune cells, such as the polarization of macrophages and the depletion of CD8+ T cells [28,29]. Currently, several studies have applied scRNA-seq technology to keloids and yielded groundbreaking findings. The aim of this review is to summarize the utilization of scRNA-seq in keloids and to highlight the compelling insights derived from this technology.

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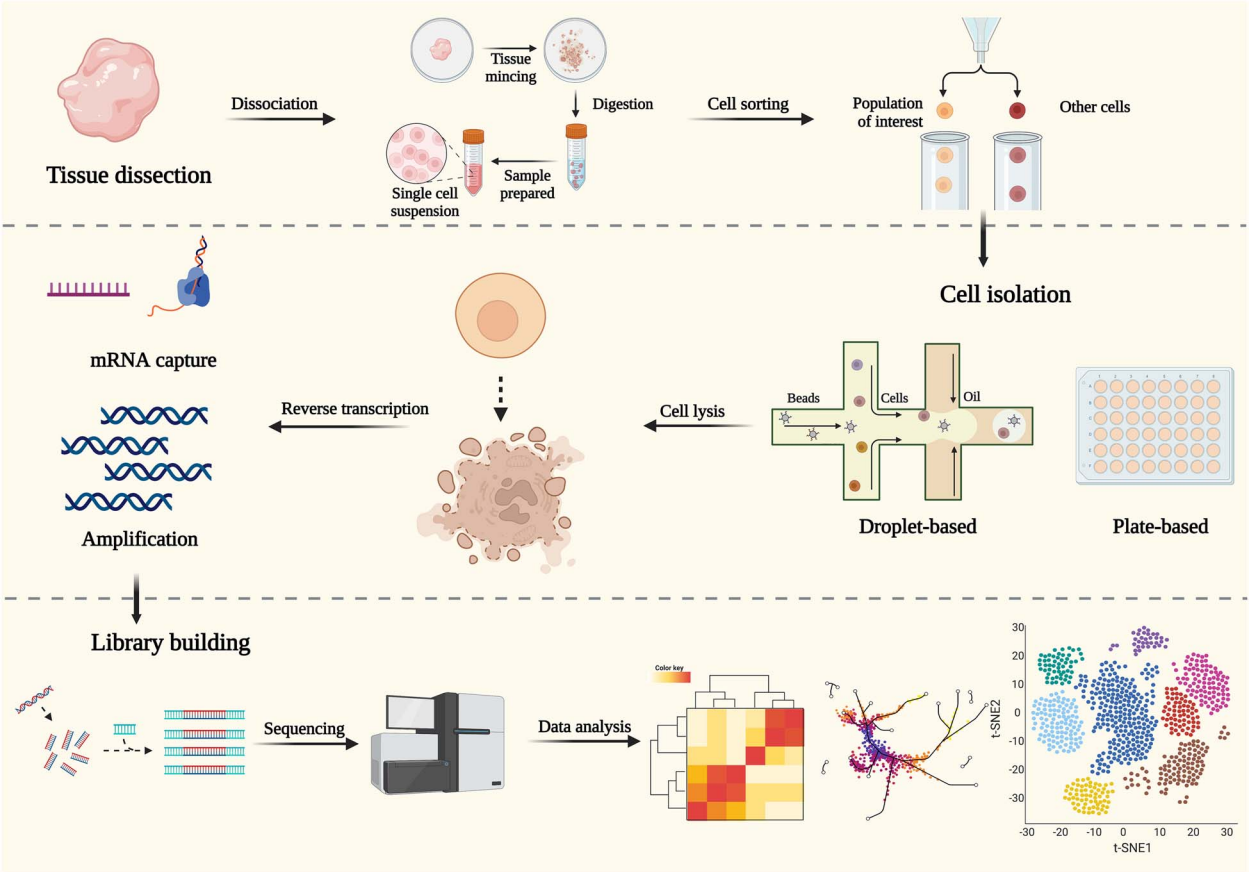


Figure 1. General pipeline of single-cell RNA sequencing. Collected samples are dissociated into single-cell suspensions. Fluorescence-activated cell sorting (FACS) and other sorting methods such as magnetic-activated cell sorting are available for enrichment of specific cell types. Cells are separated into individual wells on a 96-well plate or individual cells are captured using separate droplets. Reverse transcription and amplification of the captured mRNA is required prior to library preparation and high-throughput sequencing. The transcriptional profiling data obtained can then be pre-processed and subjected to clustering, pseudotime analysis and other downstream analyses. Created with BioRender. *POSTN* Periostin, *FN1* Fibronectin 1

Contribution of scRNA to the understanding of keloid scarring

Deciphering the exact cellular composition of keloids The complex cellular composition and intercellular crosstalk in keloids pose significant challenges to understanding their pathogenic mechanisms (Figure 2). Specifically, massive extracellular matrix deposits, fibroblasts, endothelial cells (ECs) and immune cells constitute the complex microenvironment of keloids, with concomitant transcriptomic and epigenetic alterations that pose obstacles to deciphering the exact cellular composition [30]. Traditional molecular techniques, e.g. immunohistochemistry, flow cytometry, *in situ* hybridization and quantitative polymerase chain reaction, can assist in exploring one or more cells in keloids but are extremely limited in comprehensively dissecting the cellular composition of keloids as a whole. The ability of scRNA to record transcriptomic information from individual cells and cluster cells in a massive dataset has immensely facilitated the profiling of the cellular components of keloids. Table 1 shows representative studies of scRNA analysis in keloids. Deng *et al*. performed scRNA-seq of the dermal tissue of keloids and revealed that keloids are mainly composed of fibroblasts, ECs, smooth muscle cells, keratinocytes, immune cells, lymphatic ECs, neuronal cells and melanocytes [31]. Such a cellular composition was confirmed by Direder *et al*., who further subdivided immune cells into macrophages, dendritic cells and T cells and classified the neuronal cells in keloids as Schwann cells [32]. Furthermore, the unique key roles in keloids of fibroblasts, Schwann cells and the immune microenvironment, have been thoroughly investigated from the perspective of single-cell sequencing.

Heterogeneity of fibroblasts It is widely believed that keloid scars are the result of excessive proliferation of fibroblasts and excessive deposition of extracellular matrix and therefore fibroblasts have been the centerpiece of keloid research [33–35]. scRNA-seq technology has brought data-driven innovation to understanding the pathogenesis of keloids. This technology alters the previous understanding of keloid fibroblasts and reveals the heterogeneity and multiple functional subtypes of fibroblasts involved in the skin inflammatory response and wound healing (Figure 3).

Solé-Boldo *et al*. performed scRNA on normal skin and conducted gene ontology analysis of the most representative markers in each fibroblast cluster obtained by unsupervised clustering [36]. They found specific functional enrichment

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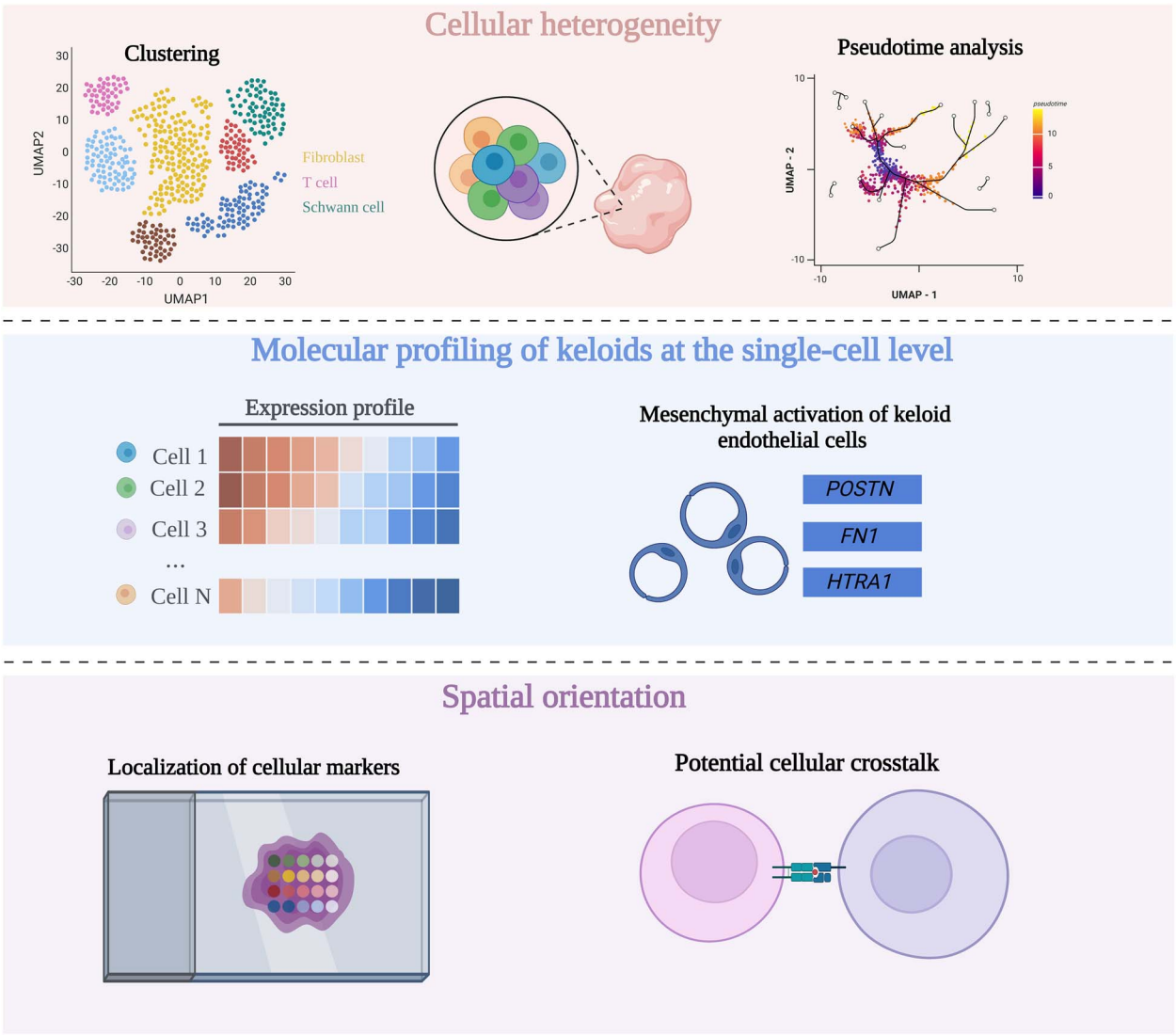


Figure 2. Application of single-cell RNA sequencing to resolve the cellular landscape of keloid. scRNA has three main applications in keloids: discovery of cellular heterogeneity, acquisition of transcriptional profiles of individual cells and validation of spatial information. Construction of sequencing libraries on individual cells allows precise differentiation of intercellular heterogeneity, clustering of cells to identify rare cell populations, and construction of trajectories of lineage development. Transcriptional analysis of individual cells can efficiently identify cell-specific gene expression, such as detecting mesenchymal activation. scRNA sequencing combined with spatial transcriptome technology enables the localization of cell-specific markers and the validation of potential cellular interactions. Created with BioRender

in certain clusters, including ossification, inflammation, cellular chemotaxis and collagen synthesis. Additionally, they combined the expression of markers in the superficial papillary dermis [representative markers are adenomatosis polyposis coli down-regulated 1 protein (APCDD1) and collagen type XVIII alpha 1 (COL18A1)] and fibroblasts in the underlying reticular dermis (representative markers are matrix gla protein (MGP) and microfibril associated protein 5 (MFAP5)) and classified dermal fibroblasts into four subgroups, namely, secretory-papillary fibroblasts, secretory-reticular fibroblasts, mesenchymal fibroblasts and proinflammatory fibroblasts. The subdivision of fibroblasts in keloids in the study by Deng *et al.* also followed this pattern [31]. To trace the intersubpopulation developmental trajectory of fibroblasts, Shim *et al.* divided keloid fibroblasts (FB) into seven subpopulations (from FB1–FB7) and revealed the gene expression patterns of fibroblasts in their different differentiation states [37]. Fibroblasts followed a differentiation direction from FB4 (the major subpopulation in normal skin) to FB1 and FB2 (the main subpopulation in keloids). The results of the enrichment analysis indicated that the FB1 and FB2 subpopulations are involved in processes related to skeletal system development, ossification and extracellular matrix proteoglycans, implying that these subpopulations have mesenchymal characteristics. In addition, the spatial localization of APCDD1 and secretory leukocyte peptidase inhibitor (SLPI) verified that keloid fibroblasts indeed possess spatial distribution characteristics from the papillary dermis to the reticular dermis.

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Table 1. Representative studies of keloid through single-cell analysis

Protocol	Samples	Molecular level of analysis	Major findings	Reference
10× Genomics	3 keloids and 3 normal scars	Transcriptomics	Keloidal fibroblasts are heterogeneous. Mesenchymal fibroblasts are increased in keloids, which are essential for collagen overexpression.	[31]
10× Genomics	4 keloids, 1 normal skin and 3 normal scars	Transcriptomics	In keloid lesions, Schwann cells have a phenotype of promoting extracellular matrix formation and influencing the polarization of macrophages.	[32]
10× Genomics	Peripheral blood mononuclear cells from 2 keloid patients and 2 healthy patients	Transcriptomics	Downregulation of cytotoxic CD8+ T cells is a critical immunological feature of keloids. The NKG2A/CD94 complex is specifically upregulated, which may contribute to the significant reduction of cytotoxic CD8+ T cells.	[76]
Singleron GEXSCOPE	12 pairs of keloids and adjacent normal skin tissues	Transcriptomics	The immune profile of keloids varies considerably from that of normal skins, and there might be a regulatory network of communication centered on macrophages in keloids.	[67]
10× Genomics	1 normal scar and 2 keloid samples (scRNA-seq) 2 keloids and 2 adjacent normal skins (spatial transcriptomics)	Transcriptomics and spatial transcriptomics	The findings in keloids at single-cell resolution have been spatially validated by spatial transcriptomics and revealed mesenchymal activation of endothelial cells.	[37]
10× Genomics	4 pairs of keloids and adjacent normal skin tissues	Transcriptomics	TGFβ signaling could be a simultaneously inhibited target for myofibril formation and angiogenesis in keloids.	[95]
10× Genomics	5 pairs of earlobe keloids and adjacent normal skin tissues	Transcriptomics	Fibroblasts, vascular endothelial cells and smooth muscle cells are defined as ECM-related populations. There are interactions between Schwann cells and ECM-related populations mediated by the SEMA3C signaling pathway and the MK/PTN gene family.	[60]

scRNA-seq single-cell RNA sequencing, *TGFβ* Transforming Growth Factor Beta, *ECM* extracellular matrix, *MK* Midkine, *PTN* Pleiotrophin; SEMA3C, Semaphorin 3C, *NKG2A* Killer Cell Lectin Like Receptor C1, *CD94* Killer Cell Lectin Like Receptor D1

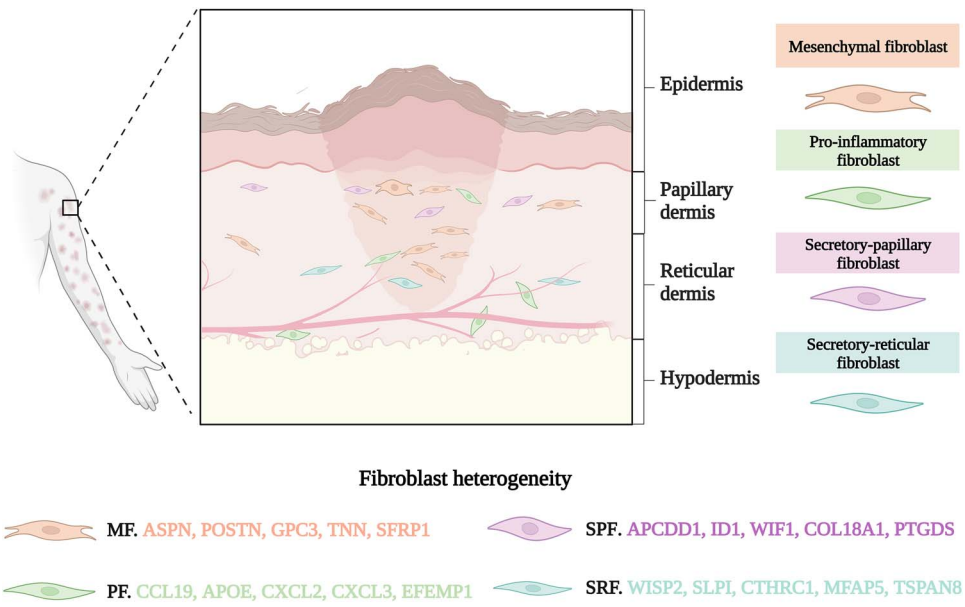


Figure 3. Heterogeneity of fibroblasts. Dermal fibroblasts can be structurally and functionally divided into four subpopulations: SRF, predominantly located in the reticular dermis; SPF, located mainly in the papillary dermis; MF, with a more reticular localization and showing greater mesenchymal potential; and PF, with a large preferential association with the vascular system and characterized by pro-inflammatory functions [28]. The subdivision of fibroblasts in keloids can follow the pattern of these four subpopulations, but with a significantly higher proportion of mesenchymal fibroblasts compared to normal skin. The area below demonstrates representative markers for the four subpopulations. *SRF* Secretory-reticular fibroblasts, *SPF* secretory papillary fibroblasts, *MF* mesenchymal fibroblasts, *PF* pro-inflammatory fibroblasts. Created with BioRender

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Interestingly, mesenchymal fibroblasts have been demonstrated to be the main enforcers of collagen overexpression, and their increased numbers may be the primary mechanism of keloid scarring. On the one hand, mesenchymal fibroblasts in keloids have a substantially increased cell ratio compared to normal scars, have more intense expression of ossification-related genes such as cartilage oligomeric matrix protein and periostin (POSTN) and collagen-related genes like collagen type I alpha 1 (COL1A1), and are enriched for osteogenic and tendon differentiation-related transcription factors including SCX, CREB3L1 and RUNX2 [31,38,39]. By flow sorting of mesenchymal fibroblasts and treatment with neutralizing antibodies against POSTN, collagen I and III expression in fibroblasts can be significantly inhibited. On the other hand, quantification of the expression of a myofibroblast marker, actin alpha 2, revealed that myofibroblasts were distributed among all four fibroblast subtypes [31,40]. However, this distribution was not homogeneous, with more than half of the myofibroblasts concentrated in mesenchymal fibroblasts [31]. Myofibroblasts have long been recognized as essential cells in the fibrogenesis process and are intermediate between smooth muscle cells and fibroblasts in terms of ultrastructure and physiological function [41,42].

Wound healing is a dynamic process highly coordinated and controlled by numerous cellular and molecular events. Mesenchymal stem cells (MSCs) are crucial contributors to extracellular matrix production and remodeling, fibroblast proliferation and macrophage polarization [43]. Persistent inflammation leads to excessive immune cell recruitment and delays skin healing, whereas MSCs can promote the polarization of macrophages toward the M2-like phenotype to moderate the inflammatory response. MSCs indirectly stimulate the migration and proliferation of keratinocytes and recruit fibroblasts through the polarization of M2 macrophages [44,45]. In addition, MSC-derived exosomes directly promote angiogenesis, keratinocyte and dermal fibroblast proliferation and migration [46]. MSCs have been proven to migrate to damaged areas and differentiate into fibroblasts and keratinocytes to fill defects and produce collagen [47]. Progressive fibrosis of the skin is a destructive process that disrupts the normal tissue architecture, manifested by the absence of dermal papillae, basement membrane and skin appendages [48,49]. Differentiation to myofibroblasts is one of the key steps in fibrosis. MSCs are considered to be a possible cell source for myofibroblasts because of their strong self-renewal ability, multidirectional differentiation potential and immunomodulatory capacity [50]. In view of this, there is an urgent necessity to track and validate the source of MSCs in the skin and the fate switch between MSCs and myofibroblasts using single cell sequencing in the future [50].

scRNA-seq reveals the molecular features of unique Schwann cell clusters Heterogeneity between patients and within lesions has hindered the exploration of the cellular and molecular mechanisms underlying keloid development and progression. The vast majority of studies have been conducted in keloid fibroblasts, and research on rare cell populations is extremely restricted. While biological techniques have limited the previous exploration of rare cell populations, the presence of unique cell populations is highly relevant to the clinical symptoms and therapeutic response of keloids. scRNA-seq is able to accomplish clustering of rare cell populations based on transcriptional similarities and define isolated cell pools into unambiguous cell clusters based on established or novel molecular features, as represented by the identification of keloidal Schwann cells [32].

Pruritus and pain are the dominant clinical symptoms of keloids; therefore, the role of neurons within the lesions is non-negligible [51,52]. Cutaneous sensory nerves reach from the superficial fascia to the dermal papillae and epidermis, forming a complex subepidermal nerve plexus [53]. In the peripheral nervous system, Schwann cells secrete neurotrophic factors, promote the survival of damaged neurons and maintain the integrity of sensory neurons. Schwann cells are not just cells committed to the glial fate [54,55]. Schwann cells have plasticity and the potential to differentiate into melanocytes and intraneural fibroblasts under specific conditions [56,57]. Moreover, the role of Schwann cells in wound healing is gradually being recognized, with their function influencing wound contraction and skin re-epithelialization and inducing the emergence of myofibroblasts through transforming growth factor β (TGFβ) signaling [58,59].

scRNA-seq revealed that the proportion of Schwann cells in keloids is significantly increased and has a transcriptional profile distinct from that in normal skin [32]. Schwann cells in keloids compared to skin exhibit significant upregulation of matrix-related genes including TGFβ inducted, tenascin-c, COL1A1, COL3A1, insulin-like growth factor binding protein 3 (IGFBP3) and insulin-like growth factor binding protein 5 (IGFBP5), suggesting their possible involvement in matrix synthesis, whereas no remarkable differences in expression of inflammatory response-related genes are observed in keloids and skin. Schwann cells in the normal skin dermis wrap around axons and mostly express genes related to myelin formation, such as myelin basic protein and myelin protein zero. Most Schwann cells in keloids do not encapsulate axons and are characterized by the expression of nestin, pro-fibrotic IGFBP5 and anti-inflammatory IGFBP3. Furthermore, keloid-specific dual-positive cells for Schwann cell marker S100 calcium binding protein B (S100B) and endothelial marker CD31 and dual-positive cells for S100B and fibroblast marker CD90 are present in keloids. Simulation of Schwann cell differentiation trajectories indicates that keloidal Schwann cells originate from differentiated myelinated Schwann cells, and the expression of genes related to myelin formation, such as neuroblast differentiation-associated protein (AHNAK) and neuronal membrane glycoprotein M6-B, decreases and gradually acquires a proliferative or dedifferentiated state, forming double-positive cells. This is paralleled by the pattern of dedifferentiation and the restoration of proliferative

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properties of myelinated Schwann cells after peripheral nerve injury. Furthermore, the cellular communication landscape of earlobe keloid suggested that Schwann cells could influence the proliferation of fibroblasts, vascular ECs and smooth muscle cells through the semaphorin 3C (SEMA3C) signaling pathway and the VEGF signaling pathway, and could also be regulated by other cell populations through midkine or pleiotrophin signaling [60].

Dissecting the immune microenvironmental architecture of keloids Keloids are histologically composed of a massive amount of irregular collagen fibers and substantial inflammatory cell infiltration. The shift in the perception of keloid scarring from a 'fibroproliferative disease' to a 'reticular dermal inflammatory disorder' demonstrates the essential role of the complicated immune microenvironment in promoting fibrosis [61,62]. Skin damage that reaches deep into the reticular dermis and the upregulation of proinflammatory factors such as interleukin 6 (IL-6) and TNF-α in keloid tissues contribute to local chronic inflammation, which may lead to invasive keloid growth [63,64]. Additionally, the function of the immune system influences the balance between proliferation and atrophy of keloids [65,66]. Although the framework of the interaction between the immune system and tumors is relatively clearly understood, the regulation of keloids by the immune microenvironment is in the preliminary stage of exploration. Single-cell sequencing provides an efficient instrument to understand the construction and lineage development of the immune microenvironment, facilitating the in-depth identification of immune cell populations with specific functions and exploring the diversity of lymphocytes and myeloid cells in keloids.

scRNA-seq parses the composition of T-cell subpopulations T cells have been implicated in tumors with dual roles in cytotoxicity and resistance to immune response, and their versatility further emphasizes the necessity to resolve the composition of T-cell subpopulations and the corresponding molecular signatures. Descending clustering analysis of scRNA-seq data of keloids has identified multiple CD4+ or CD8+ T-cell subpopulations, namely, CD4+ naive T cells, CD4+ effector memory T cells, CD8+ effector T cells, CD8+ mucosa-associated invariant T cells, CD4+ regulatory T cells and proliferative T cells [67]. Among these subsets, the proportion of CD8+ effector T cells is the highest in keloids. CD8+ effector T cells specifically express multiple genes related to cytolytic [e.g. natural killer cell granule protein 7, granzyme B (GZMB) and GZMH] and chemotactic [e.g. C-C motif chemokine ligand 4 like 2 (CCL4L2), C-C motif chemokine ligand 4 (CCL4), X-C motif chemokine ligand 2 (XCL2) and XCL1] responses. CD4+ regulatory T cells have high expression levels of typical regulatory T cell markers, including forkhead box P3, T cell immunoreceptor with Ig and ITIM domains (TIGIT), CD27 and TNF receptor superfamily member 18. CD4+ effector memory T cells have high expression levels of several heat-shock protein families like heat shock protein 90 alpha family class A member 1 (HSP90AA1). In addition to their protective functions against infection and cellular stress, the heat-shock protein family has been reported to be involved in antigen presentation, mediating the activation of monocytes and macrophages and thus promoting the innate immune response [68,69]. Additionally, scRNA-seq identified mucosa-associated invariant T cells in keloids with high expression of apoptosis-related genes (Jun proto-oncogene, AP-1 transcription factor subunit (JUN) and dual-specificity phosphatase 1 (DUSP1)). Mucosa-associated invariant T cells are tissue-resident lymphocytes that recognize metabolites of bacteria and some fungi and are activated by these antigens to express the potent cytotoxic effector molecules like interferon gamma (IFN-γ) for immune surveillance [70,71]. Collectively, scRNA-seq contributes to the resolution of the unique composition of T-cell subsets, but the function of these subpopulations and the phenotypic changes require investigation in further *in vivo* mechanistic studies.

Downregulation of cytotoxic CD8+ T cells has been determined as a cellular feature of keloid patients CD8+ cytotoxic T lymphocytes (CTLs) are typically considered to be the first choice of immune cells for targeted cancer therapy. CTLs recognize specific antigenic peptides through T-cell receptors and initiate various apoptotic pathways by releasing cytotoxic substances, such as soluble granzyme B and perforin, to tumor cells via secretory lysosomes in the presence of adhesion and costimulatory molecules [72,73]. In the context of prolonged antigen exposure, CTLs differentiate toward a 'T-cell depletion' stage, with impaired cell proliferation, reduced cytotoxic activity and decreased production of cytokines (e.g. IL-2, TNF-α and IFN-γ). Exhausted CTLs are accompanied by high expression of inhibitory receptors, including programmed cell death 1 (PD-1), TIGIT and other immune checkpoint molecules [74,75].

CD8+ CTLs were reported to be specifically downregulated in both the circulatory system and tissues of keloids, and this downregulation could be corrected by an intralesional injection of triamcinolone acetonide in combination with 5-fluorouracil [76]. The proportion of CD8+ CTLs producing granzyme B, granulysin, and IFN-γ in peripheral blood was significantly reduced in keloid patients, but the expression of inhibitory receptors, including PD-1 and LAG3, was not markedly altered. Immunohistochemical staining confirmed a decline in the number of CD8+ GZMB+ cells and an increase in the quantity of CD8+ NKG2A+ cells. NKG2A is primarily expressed on the surface of NK cells and partially on T cells and forms a heterodimeric complex with CD94 molecules (NK-cell surface membrane proteins), which are recognized by HLA-E on target cells, thereby inducing a cascade of inhibitory signals that suppress the cytotoxic activity of NK cells and CD8+ T cells [77–79]. The NKG2A-soluble HLA-E axis has been well described in keloids. The diagnostic sensitivity and specificity of soluble HLA-E for keloids are 83.69 and 92.16%, respectively, and can be considered a marker to assess the risk of keloid formation [76]. Moreover, soluble HLA-E levels correlated with treatment and recurrence reactions of keloids and possess promising

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predictive power for clinical outcomes of intralesional injection therapy.

Identification of sophisticated macrophage subtypes The monocyte–phagocyte system is the principal effector cell system participating in the immune response, consisting mainly of dendritic cells, monocytes and macrophages that maintain the homeostasis of the organism [80,81]. The subdivision of the monocyte–phagocyte system in keloids identified conventional type I dendritic cells (cDC1s), conventional type II dendritic cells (cDC2s), plasmacytoid dendritic cells (pDCs), monocytes, macrophages, mature dendritic cells, mast cells, neutrophils and proliferative cells, with a predominance of cDC2s and macrophages. cDC2s are the major dendritic cells in human blood, tissues and lymphoid organs and possess a stronger ability to synthesize IL-12, secrete a variety of cytokines including IL-8, IL-10 and TNF-α, and activate Th1, Th2, Th17 and CD8+ T cells *in vitro* compared to cDC1s, and have a wide range of immune response capabilities [82–84].

The proportion of macrophages in keloids was reported to be decreased compared to that in normal skin adjacent to the lesion, and further subdivision according to the macrophage polarization theory revealed a reduced proportion of M1-type macrophages [high expression of regulator of G protein signaling 1 (RGS1), CCL4L2, CCL3L1 and interleukin 1 beta (IL1B)] and an elevated proportion of M2-type macrophages (high expression of CD163, folate receptor beta (FOLR2) and CCL13) in keloids [67]. M1-type macrophages function as high secretors of proinflammatory factors, present antigens and initiate adaptive immune responses, with IL1B and CXCL2 as representative markers, while M2-type macrophages play an essential role in tissue healing by secreting suppressive cytokines to modulate the immune response and secreting extracellular matrix to promote wound repair, with MRC1 and CD163 as typical markers [85]. Moreover, the expression of extracellular matrix synthesis genes such as COL1A1 and secreted protein acidic and cysteine rich (SPARC) was upregulated in keloid M2 macrophages [67]. Considering the heterogeneity and plasticity of macrophages, the M1/M2 dichotomy-based approach may be generalized and inaccurate in the discrimination of macrophages, and a large macrophage population in the keloid displays a mixed M1/M2 phenotype, where IL1B, CXCL2, MRC1 and CD163 are all highly expressed [32]. Therefore, module identification of molecular features of macrophage subtypes in keloids based on a cell–cell similarity metric identified features of tumor-associated macrophages with high expression of apolipoprotein C1 (APOC1), ribonuclease A family member 1 (RNASE1) and cathepsin B (CTSB), of which APOC1 and CTSB are important factors promoting tumor metastasis [86,87]. In the tumor microenvironment, tumor-associated macrophages express anti-inflammatory cytokines, clearance receptors and angiogenic factors and possess the characteristics of M2-type macrophages [88,89]. In view of the remodeling role of macrophages in the extracellular matrix, in-depth typing of molecular markers of macrophages may provide novel guidance for understanding macrophage polarization and its role in driving fibrosis and finding new target cell populations for research.

scRNA-seq facilitates the comprehension of ECs ECs are critical players in metabolic pathways, the recruitment of immune cells and the initiation of various inflammatory skin diseases, and their dominant angiogenesis is an essential step in fibrotic diseases [90,91]. The architecture of the cutaneous vascular system is extremely delicate, with arterioles, venules and capillary collaterals differing in structure and function, and there is a dearth of knowledge about the specific characteristics of skin ECs [92]. A single-cell study collected 10 normal human skin tissues and mapped the landscape of ECs by sorting individual CD31+ CD45− cells and determined the tissue specificity of dermal ECs [93]. Endothelial cells were classified into five subtypes based on unsupervised clustering, namely, arteriole ECs, capillary ECs, postcapillary venule ECs, venule ECs and lymphatic ECs. Arteriole ECs are exposed to considerable permeability pressure and shear stress and express genes encoding gap junction proteins such as GJA5 and GJA4 and extracellular matrix proteins that contribute to vessel wall elasticity and strength such as FBLN5, FBLN2 and fibronectin 1 (FN1). In contrast, the venous wall is thin and is a major site of leukocyte extravasation, so venule EC expresses genes encoding proteins associated with leukocyte dialysis, like VCAM1 and SELP [94]. The description of the EC profile of keloids revealed similar subtypes, such as capillary ECs with high expression of lymphocyte antigen 6 family member E and CCL14, postcapillary venule ECs with high expression of selectin E and NF-kappa-B inhibitor alpha (NFKBIA), and arteriole ECs with high expression of Hes-related family BHLH transcription factor with YRPW motif 1 (HEY1) and matrix metallopeptidase 2 (MMP2) [37,95]. Furthermore, a distinct EC cluster was identified in keloids. The newly defined EC cluster was characterized by the expression of POSTN, FN1 and HtrA serine peptidase 1, which are unique transcriptional profile signatures of mesenchymal activation during wound healing [37]. Subsequent immunofluorescence experiments verified the colocalization of the mesenchymal marker POSTN and the vascular marker CD31, which further demonstrated the existence of mesenchymal activation of keloid ECs. Additionally, HIF-1α expression was increased in this particular EC cluster. HIF-1α acts as the primary hypoxia sensor [96,97], and the elevated coexpression of HIF-1α and POSTN implies a possible synergistic effect of mesenchymal activation and hypoxia during EC angiogenesis in keloids. Although mechanistic studies targeting mesenchymal activation remain to be further explored through functional experiments and clinical studies, the construction of transcriptional profiles at the single-cell level facilitates the discovery and understanding of heterogeneous ECs in keloids and provides an intriguing direction to investigate their promoting role.

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Inference and analysis of intercellular communication

scRNA-seq technology allows detailed classification of cell types at an unprecedented level of resolution. The gene expression information recorded by sequencing is an ideal material for inferring intercellular crosstalk. Intercellular communication is requisite for the coordination of individual cells' behavior with each other, and this communication often drives shifts in cellular state and function [98,99]. Dense communication networks between keloid fibroblasts, ECs and other cells were observed based on repositories of ligand–receptor interactions, especially in the TGF-β signaling pathway, which is consistent with previous studies. TGF-β is considered to be the master cytokine for fibroblast proliferation during fibrosis, and overexpression of TGF-β maintains the high level of fibroblast division and proliferation and collagen synthesis and secretion after skin injury. In the classical TGF-β/Smad signaling pathway, activated TGF-β transmits signals through the serine/threonine kinase complex, which consists of type I and type II receptors on the cell surface [100,101]. The TGFβ1–TGFβ receptor 1- and TGFβ1–TGFβ receptor 2-related signals are stronger in keloid mesenchymal fibroblasts and secretory-papillary fibroblasts, and TGFβ1–TGFβ receptor 1 signals are more intensive in pro-inflammatory fibroblasts and secretory-papillary fibroblasts than in normal scars [31]. Enhanced TGFβ1/Smad signaling contributes to abnormal collagen deposition and elevated collagen I/III ratios and the formation of abnormally cross-linked collagen fiber bundles by regulating fibroblast proliferation and apoptosis, and differentiation of fibroblasts to myofibroblasts [66]. In addition, a TGFB3–TGFBR2 interaction network exists between fibroblasts and ECs [95], and TGF-β3, which is highly homologous to TGF-β1 and acts similarly to TGF-β1, can independently induce fibrogenesis *in vivo* [102,103]. Furthermore, POSTN-ITGB5 signaling is enhanced in mesenchymal fibroblasts, and this signal plays a role in wound healing and ventricular remodeling after myocardial infarction [31]. FGF2–FGFR1 and FGF2–CD44 signaling from proinflammatory fibroblasts and secretory reticular fibroblasts is reduced, and FGF2 has been proven to act as an inhibitor of fibrosis by reducing type I collagen synthesis and hydroxyproline content [31]. Additionally, EFNB2/EPHB4 signaling is increased in the interaction between vascular ECs and fibroblasts as well as vascular ECs and smooth muscle cells, which are molecular markers of the arterial and venous vasculature, respectively [95]. Single-cell sequencing provides a data foundation for resolving intercellular communication networks. Although the role of multiple ligand–receptor pairs in fibrosis is well established, a global understanding of the changing communication network in keloid scars informs the validation of existing theories and the mining of potential therapeutic targets for the disease.

Conclusions

Since its advent, single-cell sequencing technology has been widely adopted for the exploration of multiple disease mechanisms. ScRNA-seq mapped the cellular profile of keloids and identified cellular heterogeneity, especially in fibroblasts. Moreover, fibroblast populations are dynamically transforming, and single-cell analysis reveals developmental trajectories and origins among different functional clusters, thus taking the study of keloids to a new level. At the same time, the integrated landscape of the immune microenvironment of keloids has been uncovered, and in-depth resolution of T-cell subsets and the monocyte-phagocyte system has enriched the understanding of the immune response in keloids. The identification of gene expression signatures of T cells and macrophages lays the foundation for subsequent molecular experiments. Although scRNA-seq has broken the bottleneck of keloid research and revealed novel insights, it is just the beginning. There are a number of further directions that rely on scRNA-seq to be explored. To illustrate, the transcriptomic and phenotypic alterations associated with multiple clinical treatments, such as triamcinolone injections and cryotherapy, require single-cell techniques to resolve the mechanisms driving keloid atrophy. Accurate characterization of drug-induced molecular reprogramming will pave the way for the design of more effective treatments to reduce the recurrence rate of keloids.

Abbreviations

AHNAK: Neuroblast differentiation-associated protein; APCDD1: Adenomatosis polyposis coli down-regulated 1; CCL13: C-C Motif chemokine ligand 13; CCL3L1: C-C motif chemokine ligand 3 like 1; cDNA: Complementary DNA; COL18A1 Collagen type XVIII alpha 1; CTL: Cytotoxic T lymphocyte; EC: Endothelial cell; FN1: Fibronectin 1; GZMB: Granzyme B; HEY1: Hes-related family BHLH transcription factor with YRPW motif 1; HIF-1α: Hypoxia inducible factor 1 subunit alpha; HSP90AA1: Heat shock protein 90 alpha family class A member 1; HSPA1A: Heat shock protein family A (Hsp70) member 1A; HSPA1B: Heat shock protein family A (Hsp70) member 1B; HSPD1: Heat shock protein family D (Hsp60) member 1; HSPE1: Heat shock protein family E (Hsp10) member 1; HSPH1: Heat shock protein family H (Hsp110) member 1; IFN-γ: Interferon gamma; IGFBP3: Insulin-like growth factor binding protein 3; IL1B: Interleukin 1 beta; IL-6: Interleukin 6; MGP: Matrix Gla protein; NKG2D: Type II integral membrane protein; PD-1: Programmed cell death 1; POSTN: Periostin; scRNA-seq: Single-cell RNA sequencing; TGFβ: Transforming growth factor beta 1; TIGIT: T Cell immunoreceptor with Ig and ITIM domains; XCL1: X-C Motif chemokine ligand 1.

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Authors' contributions

XYJ and WYB analyzed the data and wrote the manuscript. HY provided helpful discussion. All authors reviewed the manuscript.

Conflict of interest

None declared.

Data availability

Not applicable.

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