

Single cell deciphering of pruritic keloids: the interaction between fibroblasts and Schwann cells through the Midkine signaling

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Abstract

Background: Keloids are a common skin fibroproliferative disease that can result in severe aesthetic and functional concerns. Pruritus and pain are the most prevalent clinical manifestations of keloids. Schwann cells (SCs) variation and neuropathy within keloids contribute to these uncomfortable sensations; however the underlying mechanisms remain unclear. This study aims to explore the potential role of fibroblasts (FBs) and SCs in pruritic and pain keloids.

Methods: The activity of FBs and SCs was investigated using single-cell RNA sequencing (scRNA-seq) data of keloids. These bioinformatics analysis results were validated through *in vitro* cell culture, clinical samples, and *in vivo* experiments. The selected molecule was confirmed to be correlated with pain and itch and was subsequently used to treat cells in order to investigate its role in keloids. The *in vivo* inhibition assay was performed to evaluate its therapeutic potential.

Results: Our scRNA-seq analysis identified specific types of FBs and SCs were present in higher proportions in keloids and exhibited neurogenesis-related functions. Upon conducting an interaction analysis of these two cell types, we identified a critical molecule, Midkine (MDK), which is positively correlated with the patients' pain and itching levels. Besides, MDK treatment facilitated the proliferation of SCs and their transition to a repairing phenotype, resulting in neuronal axonogenesis. This activation of repairing SCs promoted the release of substance P from nerve fibers, leading to clinical symptoms of pain and pruritus in keloid patients. Targeting MDK effectively reduces abnormal Schwann cell proliferation and subsequently inhibits the secretion of neuropeptides that trigger pain and pruritus.

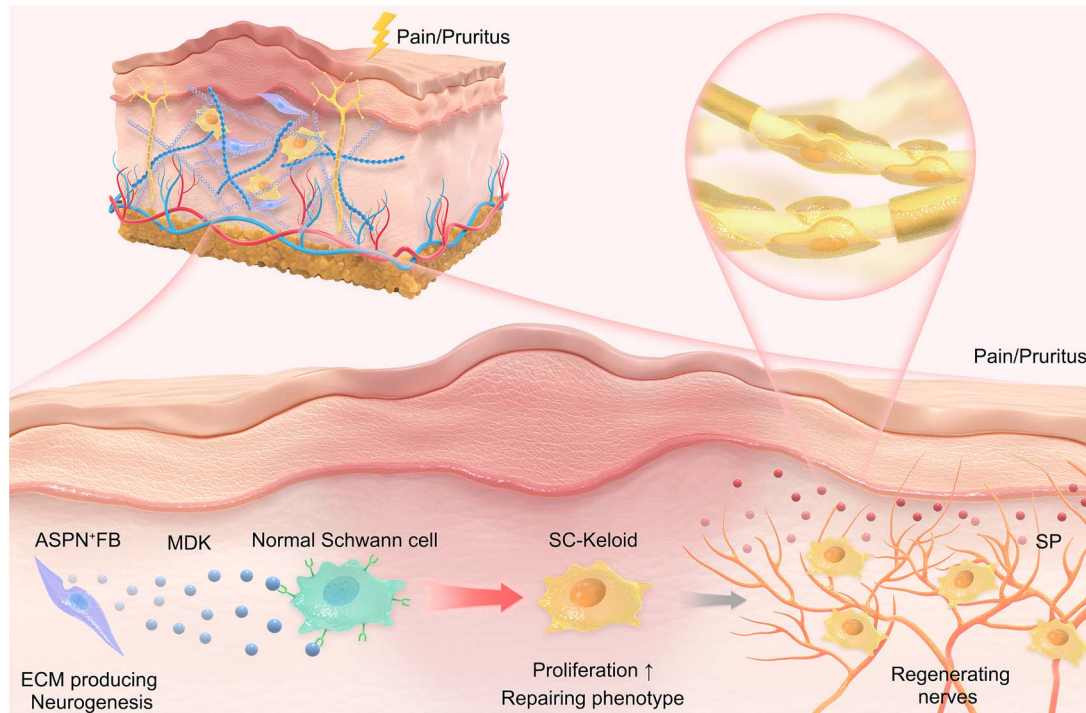
Conclusions: Our study uncovered the interaction between FBs and SCs in the development of keloidal pain and pruritus, offering a novel therapeutic strategy to alleviate the distressing symptoms of keloids.

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Graphical Abstract



Keywords: Single-cell transcriptome; Keloids; Fibroblasts; Schwann cells; Neurogenesis

Highlights

- *ASPN*⁺ FB is the dominant FB subtype in keloids with an enrichment of matrix-producing and neurogenesis functions.
- There is a subpopulation of *IGFBP5*⁺ SCs with an expansion in population and increased functions in neurogenesis and axonogenesis in keloid.
- The spatial distance between *ASPN*⁺ FBs and *IGFBP5*⁺ SCs is markedly reduced in keloids with a special activation of MDK signaling as revealed by cell–cell communication analysis.
- The expression of MDK is positively correlated with the severity of keloidal pain and pruritus in the clinical cohort of keloid patients.
- MDK promotes the proliferation of SCs and induces the transformation towards repairing phenotype. This activation of repairing SCs promotes a release of substance P from nerve fibers.

Background

Keloids are fibroproliferative dermal tumors characterized by excessive collagen deposition and infiltrative growth extending beyond the original injury site [1]. These lesions not only present significant aesthetic and functional challenges, but also induce bothersome sensory symptoms, such as pain and pruritus [2]. It is important to note a significant number of individuals diagnosed with keloids, ranging from 50% to 90%, experience these unpleasant sensations [3, 4]. Despite this prevalence, the precise mechanism responsible for the onset of pain and pruritus in keloid patients remains elusive.

The advent of scRNA-seq represents a significant advancement in the understanding of disease pathomechanisms. Recent comprehensive reviews have highlighted how this technology can resolve cellular heterogeneity and intercellular communication networks in keloid microenvironments [5]. Several research teams have already employed scRNA-seq technology to investigate keloid tissue, focusing on the cellular and transcriptional levels.

Deng et al. utilized scRNA-seq to conduct a comparative analysis of keloids and normal scars [6]. Their study classified keloidal fibroblasts into four distinct groups and observed an elevated presence of extracellular matrix (ECM)—producing

mesenchymal fibroblasts in keloid tissue. By comparing keloids with the adjacent skin using the same technology, Liu et al. [7] discovered expanded fibroblast and vascular endothelial cell subtypes, key signaling pathways like TGFβ and Eph-ephrin, along with critical regulators such as *TWIST1*, *FOXO3*, and *SMAD3*. In addition to scRNA-seq, Shim et al. also employed spatial transcriptomics technology and identified disease-related FBs in close proximity to the transcript sites of endothelial cells (ECs). ECs exhibited mesenchymal activation, which was characterized by dysregulation of TGF-β/Smad signaling [8]. Their research highlighted the role of fibrovascular communication and EC mesenchymal activation in the pathogenesis of keloids, whereas Direder et al. discovered an elevated count of phenotypically distinct Schwann cells in keloids [9, 10]. In addition to being associated with matrix formation, these keloidal Schwann cells exhibited gene expression profiles that were similar to those of repair Schwann cells found in injured peripheral nerves. However, the reason for the presence of this specific type of Schwann cells in keloids remains to be investigated.

In this study, we performed a comparative analysis on different scRNA-seq datasets of normal skin, normal scars,

keloids, and keloid-adjacent skin from four independent research groups to clarify the interactions between fibroblasts and Schwann cells. Furthermore, we screened a pivotal gene that is associated with keloid-associated pain and itching and validated it *in vitro*. Our study indicates that the pain and itching sensations in keloids are the result of a multifaceted interplay of factors within the microenvironment, which includes MDK. Consequently, the identification of key genes that are involved in the regulation of this interaction mechanism could offer crucial targets for clinical intervention in keloids.

Methods

Single cell RNA-seq data acquisition

In this study, we integrated data from four studies, totaling 29 samples (6 normal skin, 6 normal scars, 4 keloid-adjacent skin and 13 keloids). Data was downloaded from: 1. Deng et al. GSE163973. 2. Shim et al. GSE181297. 3. Direder et al. GSE181316. 4. HRA000425 dataset was kindly provided by the author Xiao Long (Figure 1a).

scRNA-seq data analysis

After being transformed into a Seurat object by the Seurat package (version 4.0.3) in R-studio (version 4.0.2, each sample's cell-gene matrix was subjected to quality control. All sample matrices were analyzed to identify 2000 highly variable genes (HVGs), which were used to integrate all matrices into one Seurat object, batch effects were mitigated, and dimensionality was reduced through principal component analysis (PCA). Cell clusters were visualized in a UMAP graph, and cell types were identified using well-established marker genes (Figure S1, see online supplementary material). Fibroblasts were reclustered at a resolution of '0.24'. Subset clusters expressing melanocyte-specific marker genes were removed. Schwann cells were reclustered at a resolution of '0.2'. To explore cell-cell interactions between cell types, the integrated scRNA-seq Seurat object was analyzed using CellChat package (version 1.6.1). Gene Ontology (GO) enrichment analysis was performed using clusterProfiler package (version 3.18.1), with p-value cutoff of 0.05. Reactome enrichment analysis was performed using Metascape (version 3.5). Gene Set Enrichment Analysis (GSEA) was plotted using <https://www.bioinformatics.com.cn>.

Clinical samples and ethics statement

A total of 21 patients aged 18 to 55 years with a confirmed clinical diagnosis of keloids for at least one year were enrolled in the study (donor information is provided in Table S1, see online supplementary material). Patients were instructed to identify and mark areas of pruritus and pain. Exclusion criteria: 1) History of neurological disorders, including alcoholism, postherpetic neuralgia, HIV infection, hypothyroidism, diabetes, or prior chemotherapy for cancer. 2) Other dermatologic condition, such as eczema, within a 5-cm radius of the keloid were excluded. 3) Patients have received systemic therapy within seven days before the study or undergone any keloid-specific treatments, including intralesional corticosteroid injections, cryotherapy, topical medications, or silicone sheet application, within six weeks prior to enrollment.

Resected keloid tissues were obtained from patients who underwent scar resection surgery, while normal skin tissue was

obtained from patients who underwent abdominoplasty. This study was approved by the Medical Ethics Committee of the Ninth People's Hospital affiliated with Shanghai Jiao Tong University School of Medicine (SH9H-2023-TK354-1), and all donors provided their written consent.

Murine cutaneous scarring model

Cutaneous scarring model was established in C57BL/6 mice under anesthesia with intraperitoneal 0.1% pentobarbital sodium (0.1 mL/10 g) [11]. Dorsal hair was removed, and the skin was disinfected. Full-thickness wounds (5 mm in diameter) extending to the superficial muscle layer were created using a biopsy punch. Mice were divided into control or inhibitors groups, the latter were treated with iMDK [12] (9 mg/kg, MCE, HY-110171A) by subcutaneous injection every 48 h. Wound healing was monitored, and early scar tissues were collected on postoperative day 14.

Mouse DRG neuron isolation and co-culture assay

DRG neurons were dissociated from DRGs of 8-week C57BL/6 mice. Euthanized mice were given cardiac perfusion with PBS. DRGs were then dissociated and digested in DMEM containing 0.1 mg/ml DNase I (Sigma-Aldrich, cat: DN25), 0.4 mg/ml trypsin type I (SigmaAldrich, cat: T8003), and 1 mg/ml collagenase type IA (Sigma-Aldrich, cat: number C9891) at 37°C for 30 min. Single-cell suspensions were plated on a glass-bottom cell culture dish (Nest Biotechnology, cat: 801001) pre-seeded with monolayer normal/MDK-treated Schwann cells. After 5 h, the medium was replaced with a neurobasal medium (Gibco, cat: 21103049,) containing B-27 supplement (Gibco, cat: 17504044), 2 mM L-glutamine (Gibco, cat: 25030081), 10% fetal bovine serum (Gibco, cat: 10099141C) and 1% antibiotic-antimycotic (Gibco, cat: 15240062,). After 72 h of co-culture, immunofluorescent staining was performed. Briefly, neurons were washed with PBS twice and fixed with 4% paraformaldehyde for 15 min, followed by blocking with 5% BSA for 1 h at room temperature. Cells were then incubated with anti- β III tubulin (Abcam, cat: ab78078, 1:200) at 4°C overnight and labeled with secondary antibodies (Invitrogen) for 2 h at room temperature. DAPI incubation at room temperature for 5 minutes was used for nuclei staining. Images were acquired using a confocal microscopy system (Leica, TSC SP8), and fields were randomly taken and calculated. Average neurite areas were quantified by Image J (version 1.53 t) and normalized to cell numbers.

Cell culture

Tissue specimens from surgery were dissected into 5 mm × 5 mm sections and immersed in 0.3% dispase II (0.3 g/ml, Gibco, 17105041) at 4°C for overnight. The epidermal layer was then removed, and the dermis was finely minced before being incubated in collagenase NB4 (3 mg/ml, Nordmark, S1745401) at 37°C for 6 h to isolate dermal FBs. The isolated FBs were cultured in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA). SCs (IMMOCELL, cat: IMP-M118) were cultured in Complete Medium (IMMOCELL, cat: IMP-M118-1). All cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. SCs were treated with DMSO (control) or MDK (5 ng/ml or 10 ng/ml) (MedChemExpress, HY-P700223AF) for 24 h.

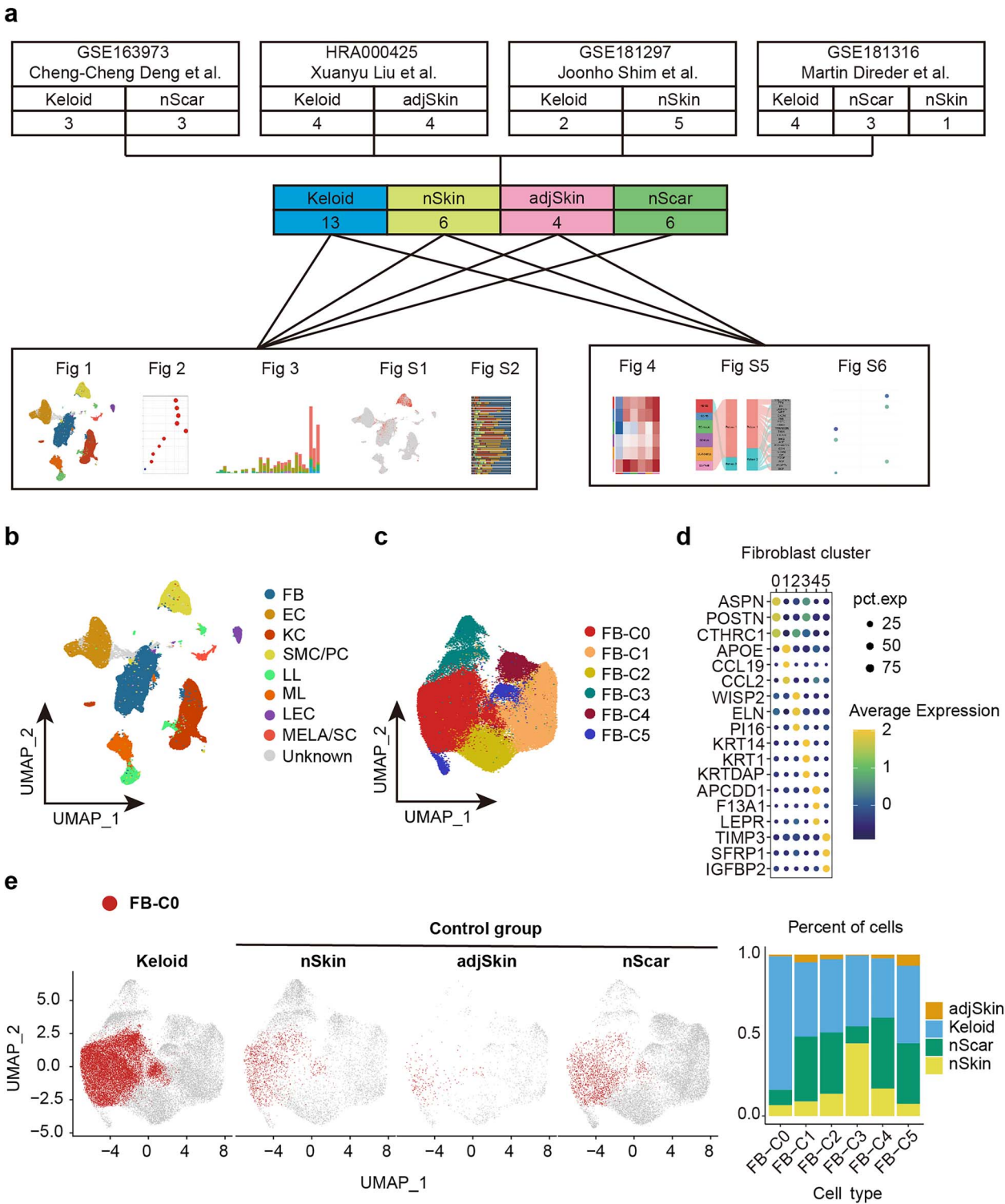


Figure 1. Single-cell RNA-seq reveals the fibroblasts subcluster of skin, keloid-adjacent skin, normal scars, and keloids. **(a)** Graphical scheme of the included datasets and bioinformatics method overview. **(b)** UMAP plot after integration of all datasets. Cluster identifications of FB, EC, KC, SMC/PC, LL, ML, LEC, unknown, MELA/SC. **(c)** UMAP-plots of re-clustered fibroblasts identified 6 fibroblast clusters. **(d)** Representative molecular signature genes for each subtype of fibroblasts. The area of the circles indicates the proportion of cells expressing the gene, while the color intensity reflects the expression level. **(e)** UMAP-plots of FB-C0 in skin, keloid-adjacent skin, normal scars, and keloids, separated by tissue. A stacked bar chart showing the proportion of 6 subtypes in 4 types of tissue. *nSkin* normal skin, *nScar* normal scar, *adjSkin* keloid-adjacent skin, *FB* fibroblast, *EC* endothelial cell, *KC* keratinocyte, *SMC/PC* smooth muscle cells and pericyte, *LL* lymphoid leukocyte, *ML* myeloid leukocyte, *LEC* lymphatic endothelial cell, *UMAP* uniform manifold approximation and projection

Cell proliferation assay

Cell proliferation was assessed using a CCK-8 kit (Dajindo, Tokyo, Japan) according to the manufacturer's protocols. In brief, cells were seeded in triplicate in 96-well plates at a density of 20 000 cells/mL. Plates were incubated at 37°C for 3–4 h after adding CCK-8 solution at the indicated time points, and absorbance at 450 nm was measured. 5-Ethynyl-2'-deoxyuridine (EdU) proliferation assay was performed using the EdU DNA Cell Proliferation kit (Beyotime Biotechnology, China) following the manufacturer's protocol.

Transwell assay

A 24-well transwell system with polycarbonate filters (8- μ m pores, Corning, NY, USA) was used for this assay. The upper compartment contained 10 000 cells suspended in the appropriate medium with 2% FBS; while the lower chamber contained 10% FBS. Following an incubation period of 1 day at 37°C, the cells in the transwell system were stained with 0.25% crystal violet. The cells remaining in the upper transwell chamber were removed, and those that migrated to the lower chamber were photographed and counted.

Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Solarbio, USA). Reverse transcription was conducted with PrimeScript RT Master Mix (Takara, RR036A). qRT-PCR was performed on an ABI QuantStudio 6 Flex system using SYBR Premix (Takara, RR066A) according to the manufacturer's instructions. The sequences of the primers are summarized in [Table S2](#) (see online supplementary material).

Western blot

Tissues and cultured cells were lysed for 30 min with RIPA lysis buffer supplemented with protease inhibitor (Roche, Mannheim, Germany). To analyze inducible protein expression, 20 μ g of protein was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Merck-Millipore, Shanghai, China). The membranes were blocked with 5% nonfat milk at room temperature for 1 hour and then incubated with primary antibodies overnight at 4°C. Subsequently, the membranes were incubated with peroxidase-conjugated secondary antibody for 1 h at room temperature before being developed with ECL western blotting detection reagent (Millipore, Billerica, MA). Immunoreactive bands were quantitatively analyzed using Image J software.

The primary antibodies used were as follows: GAPDH (1:10000, Proteintech, 60 004-1-Ig), p75NTR (1:800, Proteintech, 55 014-1-AP), and NCAM1 (1800, Proteintech, 14 255-1-AP).

Immunofluorescent staining

Tissue sections embedded in paraffin were deparaffinized, rehydrated, fixed, and blocked with 5% normal goat serum. Cultured cells were directly fixed in 4% paraformaldehyde and blocked. Next, they were then incubated with the primary antibody overnight at 4°C. On the following day, the tissue slides were incubated with secondary antibodies for 60 minutes, and nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO, USA) for

5 min. Image J software was used to analyze immunofluorescence co-localization and signal intensity of individual channels. For analysis of specific cell types and distances between different types of cells, the images were quantitatively analyzed with StrataQuest analysis software (Version 7.1.1.133, TissueGnostics GmbH, Vienna, Austria).

The primary antibodies used were as follows: α SMA (Abcam ab32575, ab7817), DAPI (SouthernBiotech), PGP9.5 (Abcam ab8189), ASPN (Affinity DF13642), MDK (Proteintech, 11 009-1-AP), S100B (Abcam ab52642), TAC1 (Proteintech, 13 839-1-AP), IGFBP5 (Proteintech, 55 205-1-AP).

Elisa

The primary fibroblast serum MDK was determined using Human Midkine ELISA Kit (Boster Biological Technology) following the manufacturer's protocol.

Cell cycle analysis

A sample of approximately 10^6 cells was collected and fixed in 75% cold ethanol at 4°C for 2 h. Subsequently, cells were stained with RNase A and propidium iodide (Cell Cycle Assay Kit, Dojindo, Japan) according to the manufacturer's instructions. Flow cytometry analysis of the samples was performed using Novo Express.

Statistical analysis

All statistical analysis were performed using R-studio (R 4.0.2) or GraphPad Prism (v9.0.0). For sequencing data analysis, the Wilcoxon rank-sum test was used to compare quantitative values between two groups. For comparisons between two groups, a Student's t-test (for normally distributed data) or a Mann-Whitney U test (for non-normally distributed data) was used. When comparing multiple groups, a one-way analysis of variance (ANOVA) was conducted when a single independent variable was present. In cases involving two or more independent variables, two-way ANOVA was employed. Prior to conducting the correlation analysis, normality tests were performed on the data. Correlation analysis between continuous variables was conducted using Pearson's correlation coefficient for normally distributed data, and Spearman's rank correlation for non-normally distributed data. All data are presented as the mean $\pm$ standard deviation (SD) as appropriate. Statistical analysis were carried out using appropriate methods to account for data distribution and ensure the validity of our conclusions.

Result

Integrated scRNA-seq reveals a dominant fibroblast subtype in keloids

In an effort to ascertain the distinctions between keloid, normal skin, normal scar and keloid adjacent normal skin at the single cell level, we integrated four previously generated scRNA-seq datasets (GSE163973 [6], HRA000425 [7], GSE181297 [8], GSE181316 [9]). In total, 174 014 cells from 29 samples were included in this study for overall analysis ([Figure 1a](#)). The data were analyzed with a standard preprocessing pipeline and cell clusters were visualized using unsupervised uniform manifold approximation and projection (UMAP) clustering. Based on well-established marker genes ([Figure S1](#), see online supplementary material),

we identified 8 distinct cell clusters, namely fibroblasts (FB), endothelial cells (EC), keratinocytes (KC), smooth muscle cells and pericytes (SMC/PC), lymphoid leukocytes (LL), myeloid leukocytes (ML), lymphatic endothelial cells (LEC), melanocytes and Schwann cells (MELA/SC) (Figure 1b). Cluster 9 was classified as an unknown group due to the unavailability of specific marker genes. In addition, these cells primarily originate from the data of Shim et al. (Figure S2, see online supplementary material), who also have difficulties in categorizing this group into specific cell types.

We then focused on FBs, which are the primary cells responsible for the progression of keloid. Six distinct clusters with specific marker genes were identified through unsupervised clustering of FBs from all samples (Figure 1c, d). Upon analyzing the proportions of each subtype of FBs in different tissue types, we discovered that FB-C0, characterized by high expression of *ASPN* and *POSTN*, consistently exhibited increased levels in all keloids compared to other samples (Figure 1e, Figure S3a, see online supplementary material).

In a previous study conducted by Deng et al, it was found that FBs subtype marked with *ASPN* and *POSTN*, displayed a mesenchymal phenotype and exhibited fibrogenesis functions [6]. We therefore propose that *ASPN*⁺ FBs may be responsible for the keloid progression and the occurrence of keloidal symptoms.

FB-C0 exhibited an enrichment of matrix-producing and neurogenesis functions

To further elucidate the biological functions of *ASPN*⁺ FBs, we performed function enrichment analysis of the up-regulated genes of this subtype. This analysis involved utilizing the reactome pathway database and performing Gene Ontology (GO) pathway analysis. The findings indicated a significant enrichment in ECM organization, crosslinking of collagen fibrils and ossification (Figure 2a-c, Figure S3b, see online supplementary material). These data suggest that one of the major functions of *ASPN*⁺ FB is ECM organization, which aligns with previous reports [6, 8, 13, 14].

Notably, we noticed a significant enrichment of neurogenesis-related pathways, including axon guidance, synapse organization, and positive regulation of neurogenesis, as determined by GO and Reactome analysis. Besides, Gene Set Enrichment Analysis (GSEA) validated the presence of an activated neurogenesis function in *ASPN*⁺ FBs, indicating that *ASPN*⁺ FBs may participate in the neuroregeneration during keloid formation and progression (Figure 2b). We further investigated the spatial correlation between *ASPN*⁺ FBs and peripheral nervous system in keloid samples. The results showed that *ASPN*⁺ FBs were aggregated around nerve fibers and their supporting Schwann cells, suggesting that *ASPN*⁺ FBs may exert their neurogenesis function by interacting with them (Figure 2d, e).

An expansion of specific Schwann cell subtypes in keloids

Schwann cells (SCs) are the predominant supporting cells in the peripheral nervous system and are critical in the process of neural repair [15]. We conducted subtype analysis and reclustered SCs into 5 separate clusters (Figure 3a). SC-Keloid was the predominant Schwann cell subtype, identified by its increased proportion in keloid. Additionally, its marker genes, *IGFBP3* and *IGFBP5*, have been reported as markers

of a Schwann cell subset involved in keloid pathogenesis [9]. SC-Nonmyel was defined by the expression of *SCN7A* and *L1CAM*, markers characteristic of non-myelinating Schwann cells, while SC-Myel was identified based on *MBP*, *MPZ*, *PRX*, and *GLDN*, which are typical markers of myelinating Schwann cells [16–18]. SC-FB was characterized by the expression of *DCN* and *COL1A1*, classical fibroblast markers, and SC-Prolif was distinguished by high levels of *TOP2A* and *KI67*, indicative of actively proliferating cells (Figure 3b). After analyzing the proportion of SC composition in each sample, we observed a significant increase in the number of SC-Keloid cells in keloids (Figure 3c, Figure S4a, see online supplementary material). In line with previous study, Direder et al. proposed that these are a group of profibrotic Schwann cells, as evidenced by their spindle-shaped morphology and lack of association with an axon. We further investigated the function of SC-Keloid using GO and GSEA analysis. The results showed that neurogenesis and axonogenesis were significantly enriched, suggesting that this cluster is actively involved in the neural repairing process during the formation of keloid (Figure 3d, Figure S4b, see online supplementary material). Immunofluorescence staining revealed a significant reduction in the spatial distance between *ASPN*⁺ FBs and *IGFBP5*⁺ SCs in keloids than normal skin and normal scar (Figure 3e). Considering that both *ASPN*⁺ FB and SC-Keloid exhibit activated neurogenesis function, we hypothesize that these two cell types may interact during the biological process of neurogenesis.

ASPN⁺ FB interacts with SC-keloid through MDK signaling

To further elucidate the interaction pattern between *ASPN*⁺ FBs and SC-Keloid, we conducted cell–cell communication analysis using CellChat [19]. Firstly, we observed an increased overall interaction between *ASPN*⁺ FBs and all SC subtypes (Figure S5a-c, see online supplementary material). More precisely, when assessing the interaction between *ASPN*⁺ FBs and SC-Keloid, we noticed that the activation of these interactions occurred in keloid when *ASPN*⁺ FBs function as the sender (Figure 4a). We then examined the specific interaction pathways between *ASPN*⁺ FBs and SC subtypes in detail. The results revealed several pathways that were specifically enriched in keloids, including *COLLAGEN*, *PDGF*, *MAG*, *LAMININ*, *MK*, *PTN*, *HSPG*, *CDH*, and *BSP*. This suggests that there is an activation of these interactions between *ASPN*⁺ FBs and SCs in the keloid tissue (Figure 4b).

Given that *ASPN*⁺ FBs may play a role in neurogenesis during keloid formation, we aimed to investigate their interaction with Schwann cells, which are key mediators of neurogenesis. To identify the critical interacting factor between them, we employed a comprehensive screening strategy that considered (1) dominant intercellular interaction pathways between *ASPN*⁺ FBs and Schwann cells, (2) genes associated with neurogenesis based on Gene Ontology analysis, and (3) genes highly expressed in *ASPN*⁺ FBs. Through this approach, we identified Midkine (MDK), a neurotrophic growth factor and a marker gene for *ASPN*⁺ FBs. Notably, MDK is also part of the MK pathway, which is significantly enriched in keloid (Figure 4c). In addition, we noticed that MK was the major outgoing signal from *ASPN*⁺ FBs when interacting with SCs, suggesting an increased secretion of MDK in *ASPN*⁺ FBs among keloid patients (Figure 4d, Figure S5d,

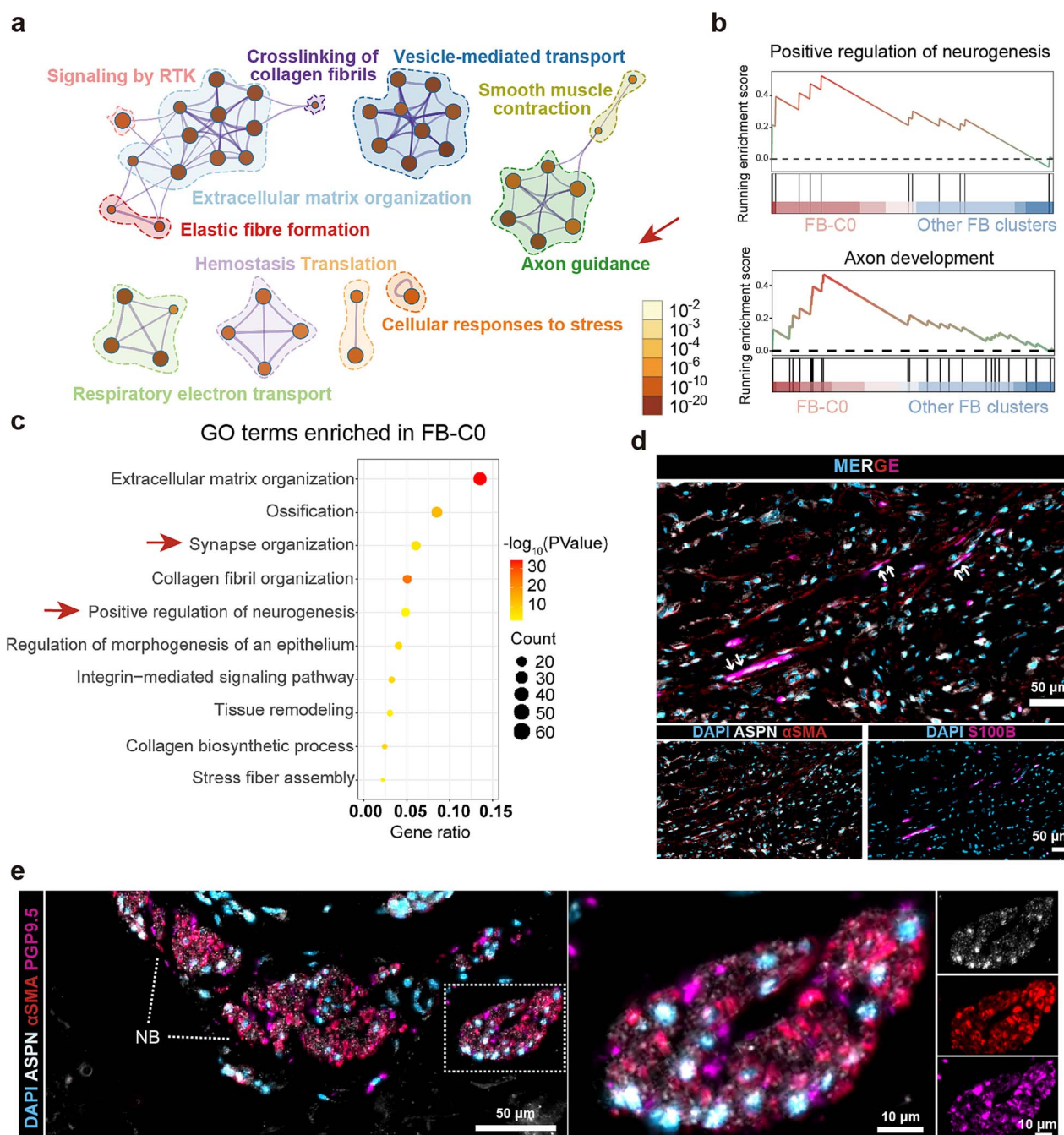


Figure 2. Identification of FB-C0 as an extracellular matrix-producing fibroblast subtype with neurogenesis function. (a) Top-ranked Reactome pathways of FB-C0 enriched genes. The arrow indicates the axon guidance pathway. (b) Gene set enrichment analysis (GSEA) revealed that FB-C0 enriched genes have a positive correlation with the positive regulation of neurogenesis and axon development pathway. (c) GO enrichment map of FB-C0 enriched genes showing the nerve system-related GO pathways (As the arrows shown). (d) Immunofluorescence staining of FB-C0 ($ASPEN^+ \alpha SMA^+$) and Schwann cells ($S100B^+$) in keloid. Double arrows indicated Schwann cells around $ASPEN^+$ fibroblasts. Scale bar: 50 μm . (e) Immunofluorescence staining of FB-C0 ($ASPEN^+ \alpha SMA^+$) and nerve fibers ($PGP9.5^+$) in keloid. NB = nerve bundle. Scale bar (left): 50 μm . Scale bar (middle and right): 10 μm . FB fibroblast, GSEA Gene set enrichment analysis, GO gene ontology

see online supplementary material). This result was further confirmed by ligand-receptor pairs analysis, which showed an increased MDK-related ligand-receptor pairs in keloid. Interestingly, the receptors of MDK including SDC4, LRP1, and ITGA6 + ITGB1 were specifically activated in the SCs of keloid (Figure 4e, Figure S6, see online supplementary material). Moreover, the expression of ITGA6 and ITGB1 was significantly increased in the SC-Keloid in keloid tissue, suggesting that SC-Keloid promotes the response to MDK

by up-regulating ITGA6 and ITGB1 during keloid formation (Figure 4f). Taken together, these findings suggest that $ASPEN^+$ FBs may interact with SCs during the neurogenesis process in keloid through MDK signaling.

MDK secreted from $ASPEN^+$ FB is positively correlated with the pain and pruritus of keloids

The expression of MDK was up-regulated in the $ASPEN^+$ FBs of keloid, as demonstrated by scRNA-seq analysis and

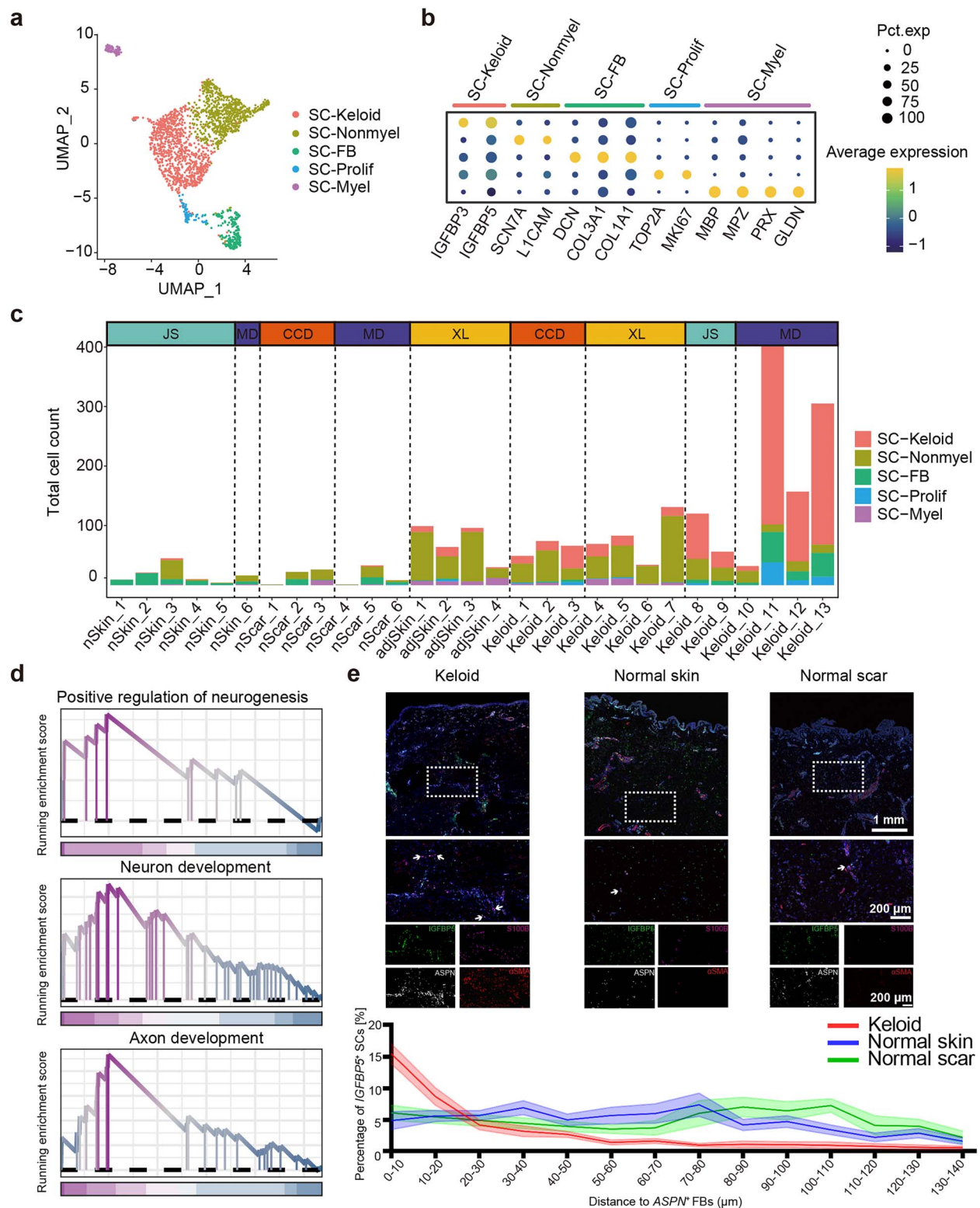


Figure 3. Schwann cell subset identifies keloid-specific Schwann cell subtypes in datasets from all sources. **(a)** UMAP plot of the Schwann cell subset (SC subset) after melanocyte removal. **(b)** Dot plot identifying the Schwann cell subcluster by expression of marker genes: IGFBP3, IGFBP5 for keloidal Schwann cells (SC-keloid); L1CAM, SCN7A for nonmyelinating Schwann cells (SC-Nonmyel); DCN, COL3A1, COL1A1 for cells expressing Schwann cell-specific and fibroblast-specific genes (SC-FB); TOP2A, MKI67 for proliferating Schwann cells (SC-Prolif); MBP, MPZ, PRX, GLDN for myelinating Schwann cells (SC-Myel). **(c)** Bar plots depicting the absolute number of distinct SC subtypes in each dataset. JS: GSE181297 from Joonho Shim et al. MD: GSE181316 from Martin Direder et al. CCD: GSE163973 from Cheng-Cheng Deng et al. XL: HRA000425 from Xuanyu Liu et al. **(d)** GSEA found that SC-keloid enriched genes are positively correlated with the positive regulation of neurogenesis, neuron development and axon development pathway. **(e)** Representative multiple immunofluorescent staining results of keloid, normal skin and normal scar samples stained for ASPN, IGFBP5, α SMA and S100B. Arrows indicated the *IGFBP5*⁺ Schwann cells. Scale bars respectively indicated 1 mm in the large-scaled images and 200 μ m in the magnified images. Below is a line graph showing distribution of the distances between *IGFBP5*⁺ SCs and *ASPN*⁺ FBs for 3 pairs of keloid, normal skin and normal scar samples, two fields of view were selected for each sample. Shaded areas show the standard error of the means. SCs Schwann cells, FBs fibroblasts, UMAP uniform manifold approximation and projection, MDK Midkine

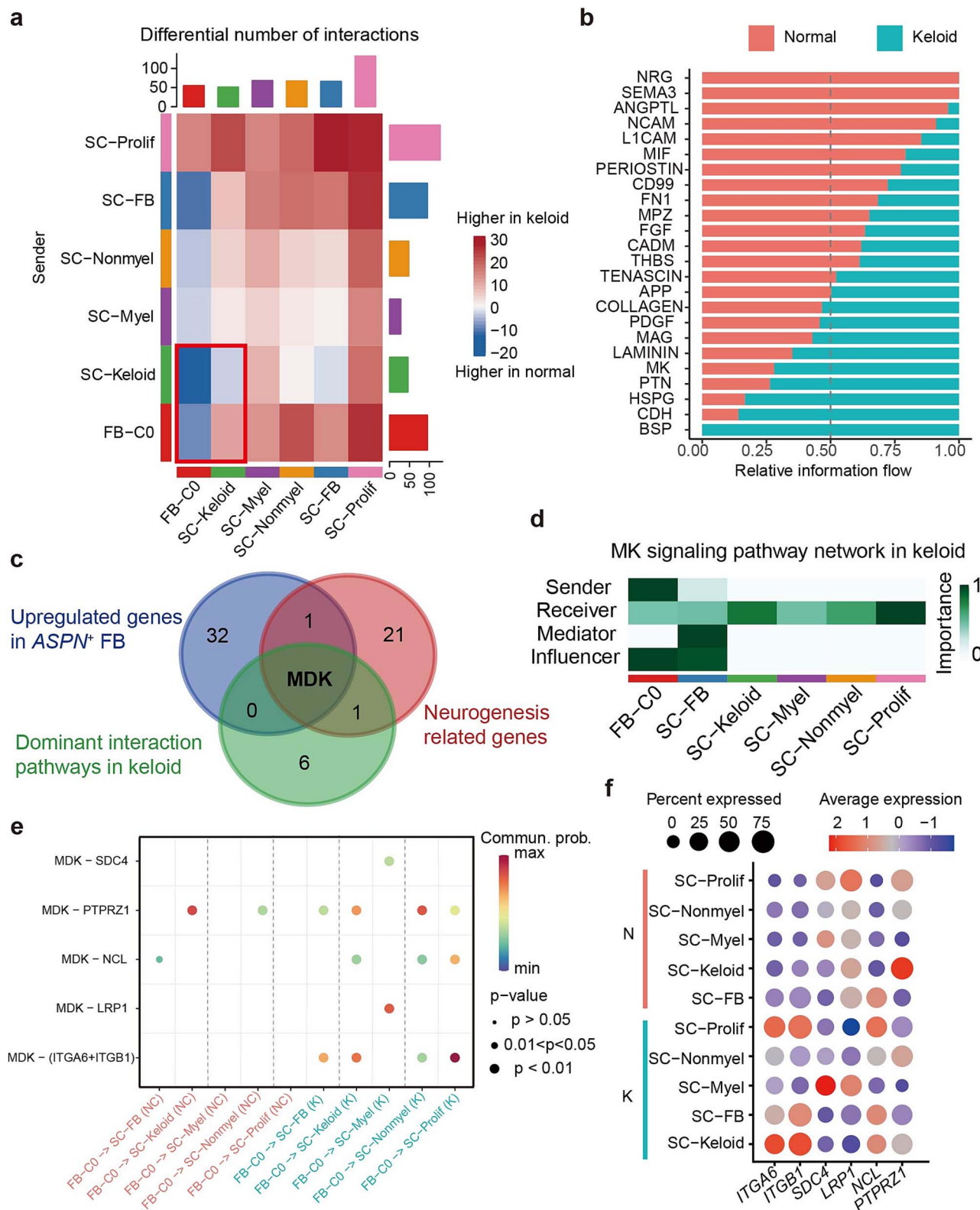


Figure 4. Cell communication between *ASPN*⁺ FBs and SCs in keloid. **(a)** Heatmap showing the number of communications between any two lineages in the FB-C0 and SC subclusters. **(b)** Stacked barplot showing the proportion of different cell communication pathways in the keloid and normal control group (nSkin and adjSkin). **(c)** Venn diagram showing the pivotal gene that mediates cell communications between FB-C0 and SC subclusters for neurogenesis. **(d)** Heatmap showing the MK signaling pathway network and the significance of each cell type in keloid. **(e)** Bubble plot of MDK-related ligand-receptor pairs in the keloid (K) and normal control groups (NC). **(f)** Dot plot of MDK receptors expression in keloid (K) and normal skin (N). The area of the circles represents the proportion of cells expressing the gene, while the color intensity represents the average expression level. SCs Schwann cells, FBs fibroblasts

immunofluorescence staining (Figure 5a, b, Figure S7, see online supplementary material). Given that MDK is a secreted growth factor, we assessed its expression in the primary fibroblasts of keloid, normal skin and normal scar. The results indicated that the expression of MDK was increased at the RNA level in the keloid fibroblasts in comparison to the normal skin and normal scar (Figure 5c). Notably, the keloid fibroblasts exhibited a higher level of MDK secretion *in vitro*, as evidenced by ELISA assay results (Figure 5d).

Pathological pain and pruritus have been reported to be associated with neuropathy [20]. Considering the potential role of MDK in the neurogenesis process in keloid as mentioned above, we evaluated the pain and pruritus symptoms in keloid patients (Information in Table S1, see online supplementary material) using the 12-Item Pruritus Severity Scale (12-PSS) and douleur neuropathique 4 questions (DN4) questionnaires [21, 22]. We then correlated the severity of the symptoms with the expression level of MDK (Figure 5e). The results showed a positive correlation between the expression level of MDK and the degree of pain and itchiness of the lesions (Figure 5f-h). These findings suggest that the MDK secreted from ASPN⁺ FB acts as a promoter and potential target for treating the pain and pruritus in keloids.

MDK promotes SCs proliferation and transformation into a repair phenotype

Given that keloid formed as a result of a dysregulated skin repairing process, we speculated that the functional status of SCs in keloid would be similar to that during neural repair. Therefore, we examined the expression of feature genes of the repairing SCs that had previously been identified in SC subpopulations [10]. Results showed that SC-Keloid exhibited a significant upregulation of these genes, indicating that SC-Keloid may be the principal SC involved in the neural repairing process during keloid formation (Figure 6a). Moreover, GSEA analysis showed that the proliferation of SC-Keloid is activated in keloid compared to normal skin (Figure 6b).

To further investigate the involvement of MDK in the functional activation and phenotype transition of SCs, we subjected SCs to various concentrations of MDK *in vitro*. Results showed that the proliferation of SCs is significantly activated upon exogenous MDK stimulation, as demonstrated by EdU (Figure 6c), CCK8 (Figure S8a, see online supplementary material) and cell cycle analysis (Figure S8b, see online supplementary material). In contrast, MDK stimulation did not interfere with the migratory capacity of SCs (Figure S8c, see online supplementary material). We validated that MDK stimulation can upregulate the expression of NCAM1 and p75NTR of SCs, resulting in a transition towards a repairing phenotype [23, 24] (Figure 6d, e). Additionally, we verified that certain factors expressed during the nerve repair program were found to be increased in MDK-treated SCs using qRT-PCR (Figure S8d, see online supplementary material). To better validate the role of MDK in neurogenesis, we performed a dorsal root ganglia (DRG) neuron-Schwann cells co-culture assay (Figure 6f). Results revealed that mouse DRG neurons cocultured with MDK treated Schwann cells exhibited denser array of neurites than those co-cultured with normal Schwann cells (Figure 6g, h). Collectively, these results suggest that MDK can induce the transition of SC to repair phenotype and facilitate axonogenesis of neuron.

The neuropeptide substance P (SP) plays an important role in the molecular bases of many human pathophysiologic processes, especially pain and pruritus [25]. Therefore, We

observed the SP expression level around nerves and Schwann cells present in the pain and pruritic keloid. The findings indicated that the regenerating nerves surrounding the SC-Keloid secrete high levels of SP, which is virtually absent in normal skin (Figure 7a). To further investigate the therapeutic potential of MDK targeting in scar-associated pruritus and pain pathogenesis, we performed a murine cutaneous scar model with MDK inhibitor (iMDK) intervention (Figure 7b). Results revealed that the iMDK treated scar sample exhibited a reduction in IGFBP5⁺ SC-Keloid subpopulation density compared to PBS control group (Figure 7c). Concurrently, SP secretion from nerve fibers significantly decreases upon MDK inhibition (Figure 7d).

Taken together, these findings suggested that MDK is involved in the expansion of IGFBP5⁺ SC-Keloid, and the release of SP from neurons in skin scarring diseases. On this basis, targeting MDK effectively reduces abnormal Schwann cell proliferation and consequently inhibits neuropeptide secretion within the nervous system.

Discussion

While numerous therapies have been developed to manage keloids, there is a lack of clinical trials specifically targeting the management of keloidal pain and itch [26]. Current treatment guidelines primarily focus on scar morphology rather than sensory symptoms [27], and while existing modalities like corticosteroids and radiotherapy have some effect on pain and pruritus, more definitive evidence is lacking to fully support their efficacy [28, 29]. One reason for these unsatisfactory outcomes is the lack of comprehensive understanding regarding the specific functions of individual cellular and molecular mechanisms [30]. Emerging characterization of the keloid microenvironment has revealed complex cellular crosstalk involving fibroblasts, Schwann cells, and immune populations [31]. The significant role of FBs in the development of keloid was acknowledged well before the advent of scRNA-seq technology. Their differentiation into myofibroblasts promotes ECM synthesis and deposition [32]. Using scRNA-seq analysis, the researchers have elucidated the role of ASPN⁺ FBs in various fibrotic diseases. In systemic sclerosis, they contribute to excessive extracellular matrix accumulation, activated by macrophages expressing TGF- β 1 [33]. In myocardial fibrosis, ASPN upregulation in cardiac fibroblasts promotes their transition into myofibroblasts and osteogenic fibroblasts via the Wnt signaling pathway, contributing to fibrosis and calcification [34, 35]. Similarly, in idiopathic pulmonary fibrosis, ASPN⁺ fibroblasts are enriched in the fibrotic niche around airways, interacting with macrophages and senescence pathways to drive disease progression [36]. Based on our findings, our study proposes, for the first time, that this subpopulation of FBs also possesses the function of neurogenesis. The presence of neuropathy within the keloid has been reported in several studies. It has been suggested that the keloid contains a greater number of nerve fibers and that damage is caused by collagen compression [37]. Following a nerve injury, FBs demonstrate upregulated expression of multiple genes and transcription factors, as well as more active interactions with other cells [38, 39]. Keloids often disrupt the patient's quality of life as a result of chronic pain and itching [40]. Some studies have shown that pain can play a role in promoting neurogenesis [41, 42]. In terms of itching, one study discovered that an inflammation-induced subset of papillary FBs promotes abnormal neurite outgrowth by

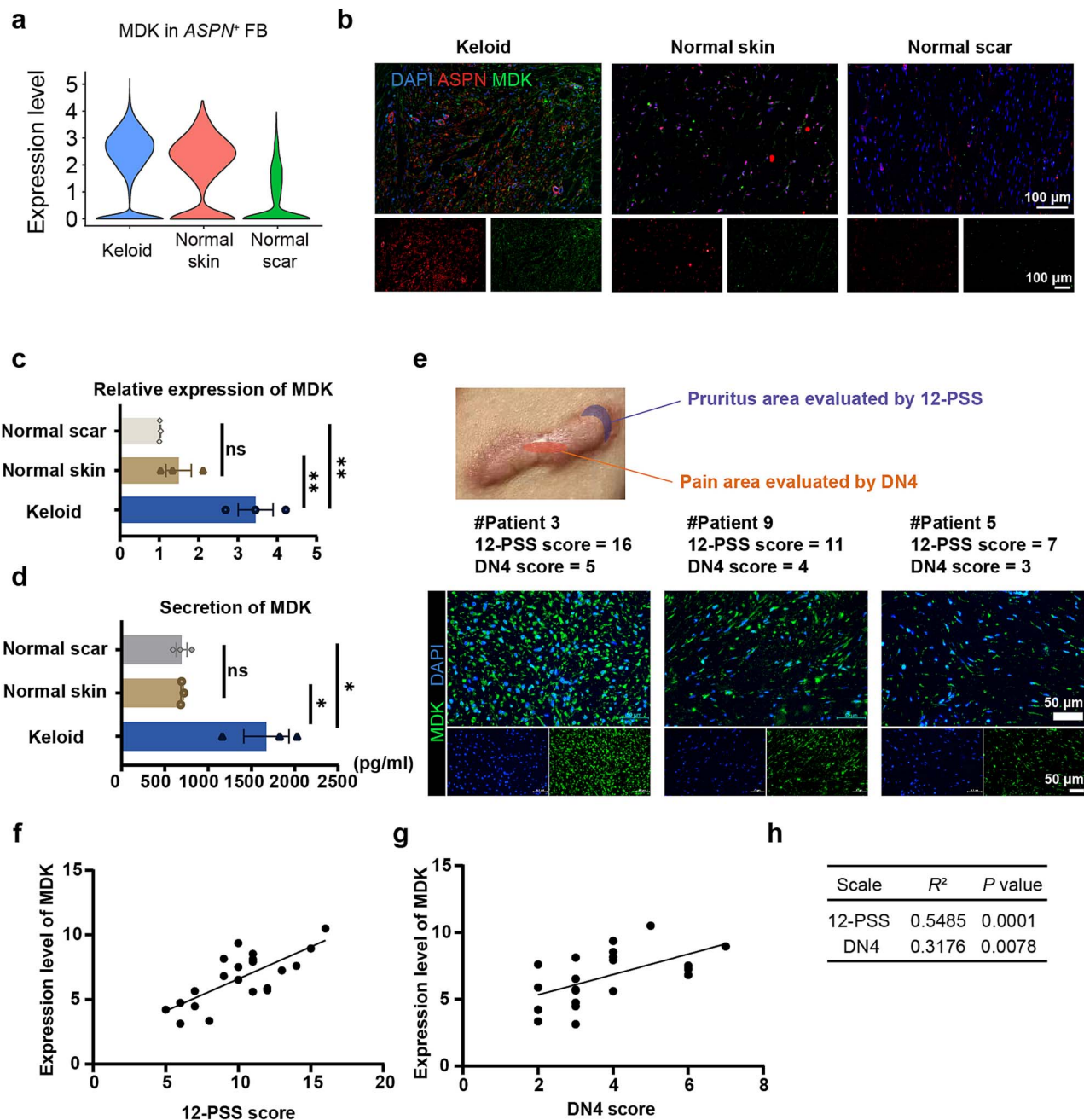


Figure 5. Comparison of MDK expression levels in keloid, normal skin and normal scar, and the correlation of MDK expression with sensory abnormalities. **(a)** Violin plot showing the expression of MDK in FB-C0 of keloid, normal skin and normal scar. **(b)** MDK- and ASPN-stained fibroblasts showed more double positive cells in keloid than normal skin and normal scar. Scale bar: 100 μ m. **(c)** Increased MDK expression level was confirmed in keloid primary fibroblasts by qRT-PCR. The data are presented as mean $\pm$ SD. the experiments were performed in triplicate, one-way ANOVA, **** $p < 0.0001$, ns, no significance. **(d)** ELISA revealed a higher level of MDK secretion level in primary fibroblasts from keloid tissue compared to normal skin and normal scar. The data are presented as mean $\pm$ SD. the experiments were performed in triplicate, one-way ANOVA, **** $p < 0.0001$, ns, no significance. **(e)** Immunofluorescence staining of samples with different scores on the 12-PSS and DN4 scales. Scale bar: 50 μ m. **(f)** Correlation analysis of 12-PSS score and MDK immunofluorescence staining intensity ($n = 21$). **(g)** Correlation analysis of DN4 score and MDK immunofluorescence staining intensity ($n = 21$). **(h)** Correlation coefficients between MDK expression level and each scale score. 12-PSS 12-item pruritus severity scale, DN4 douleur neuropathique 4 questions questionnaires

releasing the extracellular matrix protein tenascin-C (TNC) [43]. In summary, while it has been discovered that specific types of FBs possess the neurogenesis function that has been revealed in other pathological conditions, there is still a lack of research on this topic specifically with keloids.

Direder et al. proposed the presence of a distinct repair-like, profibrotic SC type in keloids [10]. However, our study

proposes, for the first time, a potential mechanism for the formation of this specific type of SC. We found that the interaction between FBs and SCs results in these specific alterations in the SC's phenotype. Numerous previous studies have demonstrated the capacity of FBs to modulate the biological effects of SCs. FBs have the ability to secrete TNC, Neuregulin, and other factors, which in turn stimulate the

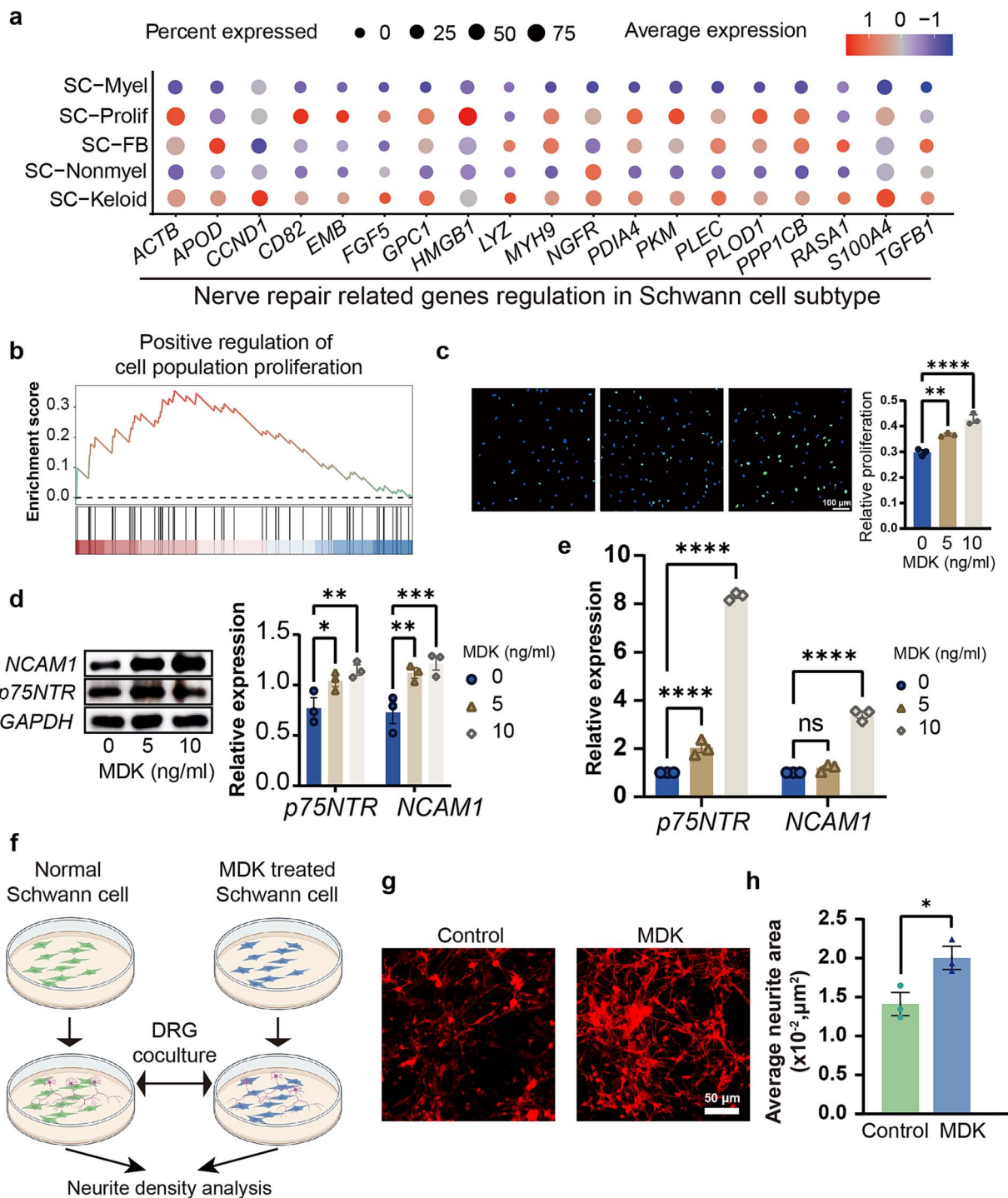


Figure 6. Characterization of SCs following MDK treatment. (a) Dotplot showing the nerve repair related genes expression in each Schwann cell subpopulation. The area of the circles indicates the proportion of cells expressing the gene, while the color intensity reflects the average expression intensity. (b) GSEA analysis revealed that in keloid, SC-keloid exhibited positive correlation with genes associated with the proliferation pathway. (c) EdU assay was used to compare the SCs' proliferation ability after treatment with MDK. Scale bar: 100 μm . (d, e) The upregulation of p75NTR and NCAM1 expression in Schwann cells following treatment with MDK was confirmed using WB (d) and qRT-PCR (e). Data are presented as mean $\pm$ SD. The experiments were performed in triplicate. Two-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns, not significant. (f) Schematic diagram of dorsal root ganglion (DRG) neuron and Schwann cells co-culture assay. (g) Representative immunofluorescent images of DRG neuron (stained by β III tubulin). Scale bar: 50 μm . (h) Quantification of average neurite area per cell in the DRG neuron and Schwann cells co-culture assay. Data are presented as mean $\pm$ SD. The experiments were performed in triplicate. T test, * $p < 0.05$. Control, mouse DRG neurons cocultured with normal Schwann cells. MDK, mouse DRG neurons cocultured with MDK treated Schwann cells. SC Schwann cell.

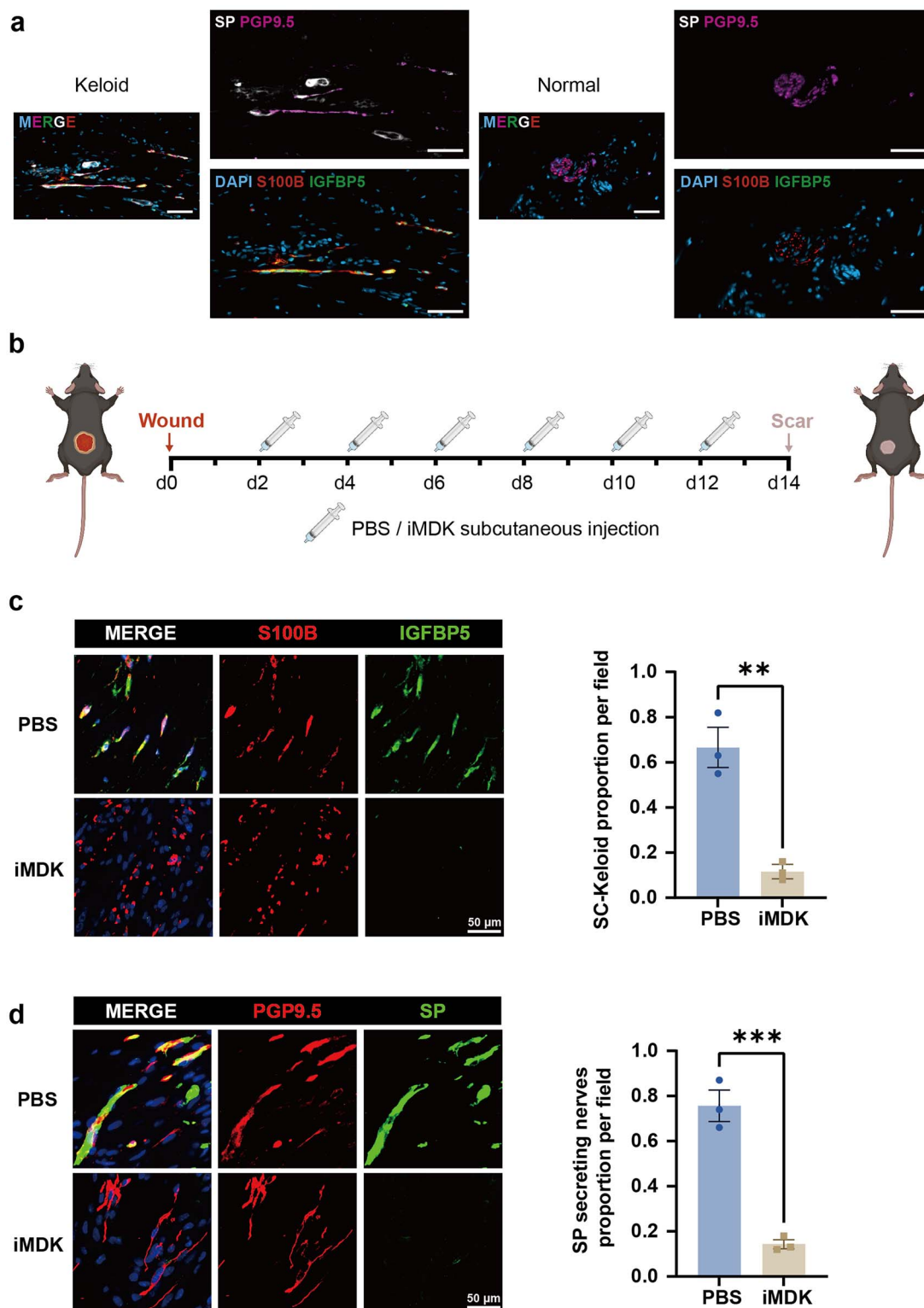


Figure 7. MDK inhibition reduces SC-keloid proliferation and SP secreting nerve fibers. **(a)** Immunofluorescence staining of SC-keloid ($S100B^{+}$ $IGFBP5^{+}$) and nociceptive nerve fibers ($PGP9.5^{+}$ SP^{+}) in keloid and normal skin. Scale bar: 50 μ m. **(b)** Schematic diagram of murine cutaneous scar model with iMDK/PBS intervention. **(c, d)** immunofluorescence staining of SC-keloid ($S100B^{+}$ $IGFBP5^{+}$) (C) and nociceptive nerve fibers ($PGP9.5^{+}$ SP^{+}) (D) in PBS and iMDK groups. Scale bar: 50 μ m. Data are presented as mean $\pm$ SD. The experiments were performed in triplicate. T test. ** $p < 0.01$, *** $p < 0.001$. SP Substance P, iMDK MDK inhibitor

migration of SCs [44, 45]. The ephrin-B/EphB2 signaling between FBs and SCs results in cell sorting, followed by the directional collective cell migration of SCs out of the nerve stumps to guide regrowing axons across the wound [46]. All of this evidence implies the presence of numerous interacting pathways and pivotal molecules between FBs and SCs, specifically in our case, a molecule called MDK.

MDK is a retinoic acid-inducible heparin-binding growth factor that possesses a wide range of functions [47]. The MDK-encoded protein promotes cell proliferation, migration, and angiogenesis, especially during tumorigenesis [48]. As a cytokine with neurotrophic and neurite outgrowth properties, MDK has also been extensively studied for its role in repairing the nervous system following injury [49]. Our co-culture assay of DRG neurons with MDK-treated Schwann cells demonstrated that MDK promotes Schwann cell phenotype transformation, which facilitates axonal growth. These findings provide functional evidence that MDK is not only involved in Schwann cell proliferation but also contributes to the neurological dysfunction of peripheral nerves within the keloid microenvironment.

There has been no prior research indicating a possible link between MDK and abnormal skin sensations. Our study proposes, for the first time, that MDK may be involved in the pathological mechanism of pain and itching in keloids. Nattkemper et al. utilized primate models to generate gene expression data that compared healthy skin with atopic and psoriatic skin lesions [50]. Upon analyzing the data, we discovered that MDK expression was elevated in primate skin affected by itching, which serves as additional evidence to support the findings of our study. Many studies have examined MDK as a therapeutic target for tumors, and there are some ongoing investigations into the use of MDK inhibitors [51–53]. Our *in vivo* data from the murine scar model revealed that pharmacologic inhibition of MDK led to a marked reduction in the *IGFBP5*⁺ SC subpopulation and a decrease in SP secretion from nerve fibers. This highlights MDK's potential role in mediating neurogenic inflammation and sensory abnormalities, such as pain and pruritus, which are often reported in keloid patients. However, further data from *in vivo* and clinical trials are still needed. Our findings present a novel therapeutic approach for drugs that target MDK to alleviate and manage the painful and itchy symptoms of keloids.

Conclusions

This study enhances our understanding of the role of FBs and SCs in the development of keloid and the itch and pain sensations associated with keloids. We identified *ASPN*⁺ FBs as possessing the ability to produce ECM and promote neurogenesis. This group of FB secretes MDK, which has an impact on SCs by stimulating their proliferation and facilitating them to transform into a reparative phenotype. As a result, the regenerating nerves release neuropeptides, which ultimately cause pain and itching in keloids. Our experiments demonstrated that MDK is a pivotal gene for the interaction between fibroblast and Schwann cell in keloids. This discovery has the potential to revolutionize the field of keloid treatment and drug discovery.

Abbreviations

SCs: Schwann cells; FBs: fibroblasts; scRNA-seq: single-cell RNA sequencing; MDK: Midkine; UMAP: uniform manifold

approximation and projection; EC: endothelial cells; KC: keratinocytes; SMC: smooth muscle cells; PC: pericytes; LL: lymphoid leukocytes; ML: myeloid leukocytes; LEC: lymphatic endothelial cells; MELA: melanocytes; ASPN: Asporin; POSTN: Periostin; ECM: extracellular matrix; GO: Gene Ontology; GSEA: Gene Set Enrichment Analysis; IGFBP3: insulin-like growth factor binding protein 3; SCN7A: sodium voltage-gated channel Alpha Subunit 7; DCN: decorin; TOP2A: DNA topoisomerase II alpha; MBP: myelin basic protein; TNC: tenascin-C

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Author contributions

E.Y, R.Q.X and L.Y.T conceptualized research; X.H and T.Z supervised research and acquired funding; E.Y performed scRNA-seq data integration and analysis; R.Q.X, prepared tissue samples and performed validation experiments; S.Y.L and Y.H.L contributed reagents and analytic tools; H.R.Z, and H.L performed visualization and figure design; S.C.G and Y.X.Z participated in data interpretation and drafted the manuscript; X.H revised the manuscript. All the authors reviewed and approved the final version of the manuscript.

Supplementary data

Supplementary data is available at *Burns & Trauma Journal* online.

Ethics statement

This study was approved by the Medical Ethics Committee of the Ninth People's Hospital affiliated with Shanghai Jiao Tong University School of Medicine (SH9H-2023-TK354-1), and all donors provided written informed consent.

Conflicts of interest

The authors declare no competing interests.

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Data availability

The data discussed in this study have been either deposited in NCBP's Gene Expression Omnibus (GEO), and are accessible through GEO series accession number GSE163973 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE163973>), GSE181297 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE181297>), GSE181316 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE181316>), or deposited in Genome sequence Archive with accession number HRA000425 (<https://ngdc.cncb.ac.cn/gsa-human/browse/HRA000425>).

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