

ZNF469 promotes extracellular matrix production in normal and keloid dermal fibroblasts

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Abstract. Aberrant extracellular matrix (ECM) production by dermal fibroblasts drives fibrotic skin diseases, which has an adverse impact on the lives of patients. Current treatments are limited; therefore, the development of new antifibrotic strategies is necessary. The aim of the present study was to investigate zinc finger 469 (ZNF469) as a potential ECM regulator in skin fibrosis. ZNF469 was knocked down in dermal fibroblasts using doxycycline-induced short hairpin RNA. ZNF469 knockdown was found to impair proliferation, migration, contraction and collagen production in dermal fibroblasts. In addition, RNA sequencing revealed that ZNF469 knockdown suppressed the expression of genes associated with the ECM and collagen biosynthesis, indicating that ZNF469 plays a role in ECM regulation. Analysis of publicly available RNA-sequencing data from hypertrophic scars and keloids revealed the upregulation of ZNF469 expression in these tissues and the positive correlation of ZNF469 expression with that of ECM-related genes. Single-cell analysis of keloids indicated that ZNF469 is localized in mesenchymal fibroblasts, a key collagen-producing fibroblast subpopulation. Pseudotime analysis suggested that ZNF469 plays a role in the establishment of this phenotype. Consistent with this notion, the knockdown of ZNF469 in dermal fibroblasts was shown to downregulate the expression of mesenchymal markers. In addition, it reduced the proliferation, migration, contraction and collagen production of keloid fibroblasts. These findings indicate that ZNF469 is a crucial regulator of ECM production and the mesenchymal fibroblast phenotype, suggesting it may be a potential therapeutic target for skin fibrosis.

Introduction

The skin is a vital organ that provides thermal regulation and physical protection against external threats (1). It is composed of three layers, namely the epidermis, dermis and hypodermis, each with distinct properties responsible for protection, structural support and energy storage, respectively. The dermis houses fibroblasts, the primary cells responsible for synthesizing and organizing the extracellular matrix (ECM), including collagen, elastin and proteoglycans (2,3). During homeostasis, fibroblasts maintain a delicate balance of ECM synthesis, which is essential for preserving tissue structure and supporting wound repair (3). However, disruption of this balance can lead to excessive ECM production, driving fibrotic skin diseases such as scleroderma, keloids and hypertrophic scars (4). These conditions, characterized by excessive collagen deposition, affect >100 million individuals annually in the developed world, causing both cosmetic disfigurement and substantial physical and psychological distress (4,5). Despite the prevalence of dermal fibrosis, current treatment strategies remain limited, underscoring the urgent need to elucidate the molecular mechanisms controlling fibroblast-mediated ECM production to develop effective anti-scarring therapeutics (6).

The ECM is a dynamic and complex network of proteins, primarily produced by fibroblasts, that provides structural support and regulates cellular behavior (7). Comprising >300 proteins, the ECM includes the core matrisome, consisting of collagens, elastic fibers, glycoproteins and proteoglycans, as well as matrisome-associated components such as ECM-modifying enzymes and growth factors (8). This intricate network plays a crucial role in skin development, aging, wound healing and tissue homeostasis (8). However, disruptions in ECM composition and organization contribute to the pathogenesis of various diseases, including skin fibrosis, where excessive collagen crosslinking leads to tissue stiffening and impaired function (9-11).

Transcription factors, a class of promoter-binding proteins, activate or repress gene expression, thereby determining cell identity and function (12). Although several transcription factors have been indicated to help maintain the fibroblast state in both healthy and pathological conditions, a comprehensive understanding of the specific transcriptional regulators of ECM-associated genes in fibroblasts remains elusive (13).

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Emerging evidence suggests that zinc finger 469 (ZNF469) is a promising positive regulator of ECM production (14-22). ZNF469, a member of the Cys2-His2 zinc finger protein family, is highly conserved across vertebrates (14). Loss-of-function mutations in ZNF469 are associated with brittle cornea syndrome (BCS) and other ECM-related diseases, underscoring its importance in ECM regulation (14-17). Zebrafish and mouse models with ZNF469 knockout exhibit phenotypes consistent with BCS, further supporting its role in collagen synthesis and ECM modulation (18,19). In addition, the RNA-sequencing (RNA-seq) analysis of keloid tissues has identified ZNF469 as a potential core regulator of the cartilage-like ECM composition in these scars (20). Furthermore, a study of human skin fibroblasts, along with our previous study in hepatic stellate cells, have shown that ZNF469 knockdown impairs collagen production, implicating its role as a profibrotic factor (21,22).

Although previous research has indicated a strong association between ZNF469 and ECM production in fibroblasts, to the best of our knowledge, no in-depth molecular studies have been conducted on this, particularly in the context of skin fibrosis. Therefore, in the present study, the aim was to comprehensively analyze the functions of ZNF469 in normal fibroblasts. In addition, keloids were used as a model of skin fibrosis to clarify the mechanisms by which ZNF469 regulates ECM production and contributes to fibrotic disease.

Materials and methods

Cell culture and lentiviral transduction. Normal and keloid fibroblasts were cultured in standard 2-dimensional conditions, strictly following the culture protocols provided by the manufacturer to maintain consistent cell characteristics. Normal and keloid human fibroblast cell lines [CCD-1064Sk, cat. no. CRL-2076; and KEL FIB, cat. no. CRL-1762, respectively; American Type Culture Collection (ATCC)] were cultured in Iscove's Modified Dulbecco's Medium (cat. no. 30-2005; ATCC) supplemented with 10% fetal bovine serum (FBS; cat. no. A5256701; Gibco; Thermo Fisher Scientific, Inc.), 100 μ g/ml Primocin (cat. no. ant-pm-05; InvivoGen), 1% Antibiotic-Antimycotic (cat. no. 15240062; Gibco; Thermo Fisher Scientific, Inc.) and GlutaMAX (cat. no. 35050061; Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂. The 293FT cell line (cat. no. R70007; Invitrogen; Thermo Fisher Scientific, Inc.) was cultured under the same conditions, with the exception that Dulbecco's modified Eagle's medium (cat. no. 11965-092; Gibco; Thermo Fisher Scientific, Inc.) was used as the basal medium.

Two short hairpin RNAs (shRNAs) targeting ZNF469 transcripts (sh1-ZNF469 and sh2-ZNF469), as well as a non-targeting scramble control shRNA, were designed using the GPP Web Portal tool (<https://portals.broadinstitute.org/gpp/public>) and inserted into an AgeI/EcoRI-cut Tet-pLKO-puro lentiviral vector (Plasmid #21915; Addgene, Inc.). The cloning primer sequences used for vector construction are presented in Table SI. Lentiviral particles were produced in 293FT cells using the second-generation packaging plasmids pMD2.G (plasmid #12259; Addgene, Inc.) and psPAX2 (plasmid #12260; Addgene, Inc.). In each 6-well plate, 293FT cells received co-transfection of 3.08 μ g Tet-pLKO-puro containing cloned shRNA, 3.08 μ g psPAX2 and 2 μ g pMD2.G using a calcium

phosphate transfection kit (cat no. CAPHOS; Sigma-Aldrich) for 8 h at 37°C. A total of 48 h post-transfection, lentiviral particles were collected and concentrated before being used to transduce normal and keloid fibroblasts at a multiplicity of infection of 5 by spinoculation (1,000 x g, 40 min, 37°C), as previously described (23). Transgenic cell lines were established by selection with puromycin (0.5 μ g/ml; cat. no. P8833; Sigma-Aldrich) and maintained under the same conditions. The Tet-pLKO-puro lentiviral vector is a tetracycline-inducible shRNA system (24). Therefore, doxycycline (2 μ g/ml; cat. no. D5207; Sigma-Aldrich), a tetracycline derivative, was used to induce shRNA expression. Cells were treated with doxycycline for 4 days prior to most of the experiments to ensure effective ZNF469 knockdown.

mRNA expression analysis. Total RNA was isolated from the cells, and cDNA was synthesized using standard reverse transcription (RT) procedures, as previously described (23). Quantitative PCR (qPCR) was then performed to analyze mRNA expression levels using 4X CAPITAL qPCR Green Master Mix (cat. no. BR0501902; Biotech Rabbit GmbH), which contains SYBR Green as a fluorophore. Thermocycling conditions included an initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Melt curve analysis was subsequently carried out at 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec. Relative gene expression was calculated using the $2^{-\Delta\Delta C_q}$ method (25), with ribosomal protein L19 serving as the reference gene for normalization (26). A list of qPCR primers, designed using PrimerQuest software (version 2.2.3; <https://sg.idtdna.com/pages/tools/primerquest>), is provided in Table SI.

Proliferation assay. Cell proliferation was assessed using the colorimetric MTT assay (cat. no. M6494; Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, cells were seeded into 96-well plates and incubated for 24, 48 or 72 h. At each time point, 0.5 mg/ml MTT in basal medium was added to each well, and the plates were incubated for 2 h at 37°C. The resulting formazan crystals were then solubilized with 100 μ l DMSO, and the absorbance was measured at 570 nm using a Synergy HTX Multi-Mode Microplate Reader (BioTek; Agilent Technologies, Inc.). The doubling time (T_d) was calculated using the following equation: $T_d = \frac{\ln 2}{B}$, where B represents the exponential growth rate constant, derived from the slope of the linear portion of the plot of the natural logarithm of absorbance values vs. time.

Migration assay. Cell migration was assessed using the Transwell assay (27). Briefly, cells were starved in FBS-free media for 24 h. Then, 1×10^5 cells in FBS-free medium were added to the upper chamber of Transwell inserts with 8- μ m pores (cat. no. 353097; Falcon; Corning Life Sciences), while the lower chamber contained complete growth medium. After incubation at 37°C for 24 h, the cells that had migrated to the lower surface of the inserts were stained with crystal violet for 10 min at room temperature. Images were captured using the brightfield mode of the EVOS M7000 Imaging System (Invitrogen; Thermo Fisher Scientific, Inc.). The number of migrated cells was counted using ImageJ software (version 1.54g; National Institutes of Health), and

cell migration was presented relative to that in the control (non-doxycycline-induced) cells.

Collagen gel contraction assay. A collagen gel contraction assay was performed as previously described (28). Briefly, a mixture of 1×10^5 cells in 0.6 ml of 1 mg/ml rat tail type I collagen (cat. no. 06-115; Merck KGaA) was prepared and neutralized with NaOH. Then, 0.5 ml of the mixture was transferred into each well of a 24-well plate. After 20 min, the solidified gel was carefully detached from the well using a P200 pipette tip. Images were captured at 24 h using the brightfield mode of the UVP ChemStudio instrument (Analytik Jena AG). The gel area was measured using ImageJ software (version 1.54g; National Institutes of Health), and collagen gel contraction was reported relative to that in the control cells.

Collagen secretion assay. Cells were cultured in 24-well plates in culture medium supplemented with 20 μ M L-ascorbic acid (cat. no. A4403; Sigma-Aldrich) for 4 consecutive days. The total amount of collagen secreted into the cell culture supernatant was assessed using the Sirius Red Collagen Detection Kit (cat. no. 9062; Chondrex, Inc.), according to the manufacturer's instructions. A 0.5-ml sample of the culture medium was collected and concentrated overnight using Concentrating solution (cat. no. 90626; Chondrex, Inc.). The concentrated samples and standards were mixed with 0.25 ml Sirius red solution for 10 min at room temperature, allowing collagen to bind to the dye. After centrifugation at $8,600 \times g$ for 3 min at room temperature to pelletize the collagen, the collagen pellet was resuspended in 0.1 ml of the extracted solution and then transferred to a 96-well plate. Absorbance was measured at 530 nm using a Synergy HTX Multi-Mode Microplate Reader, and the results were reported relative to that in the control cells.

Western blot analysis. Protein lysates were prepared as previously described (28). Briefly, cells were lysed in RIPA buffer (cat. no. RB4475; Bio Basic, Inc.), and protein concentrations were determined using the Bradford assay. Subsequently, 40 μ g protein was loaded into each lane and separated by SDS-PAGE on 8% gels, followed by protein transfer, as previously described (29). Protein-bound nitrocellulose membranes (cat. no. 10600012; Cytiva) were blocked for 5 min at room temperature with BlockPRO 1 Min Protein-Free Blocking Buffer (cat. no. BM01-500; Visual Protein; Energenesis Biomedical Co., Ltd.) and then incubated overnight at 4°C with the following primary antibodies: Rabbit polyclonal ZNF469 (1:500; custom generated) and mouse monoclonal GAPDH (1:2,000; cat. no. 437000; Invitrogen; Thermo Fisher Scientific, Inc.). The custom ZNF469 antibodies were generated by Synbio Technologies. The company synthesized a ZNF469 peptide corresponding to amino acids 1,659-1,749 of human ZNF469 in *Escherichia coli* and used it to immunize rabbits over five rounds. Serum was then harvested for antibody purification. The same peptide sequence was used by Thermo Fisher Scientific, Inc. for their discontinued antibody (cat. no. PA5-67072) and its current replacement (cat. no. PA5-145175), which were not available at the time of the study. After washing with Tris-buffered saline with 0.05% Tween 20 (cat. no. 655204; Sigma-Aldrich; Merck KGaA),

the membranes were incubated with HRP-conjugated goat anti-rabbit or anti-mouse IgG (1:5,000; cat. nos. 7074 and 7076, respectively; CST Biological Reagents Co., Ltd.) for 1 h at room temperature. Following additional washes, chemiluminescent detection was performed using ECL Select Western Blotting Detection Reagent (cat. no. RPN2235; Cytiva). Blots were scanned using a UVP ChemStudio instrument.

Immunofluorescence (IF) assay. Cells were cultured for 4 days prior to harvesting, then fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS, and blocked with 1% bovine serum albumin (cat. no. BSA-1S; Capricorn Scientific) in PBS for 1 h at room temperature. The cells were then incubated overnight at 4°C with primary antibodies against type I collagen (1:1,000; cat. no. 8-3A5; Developmental Studies Hybridoma Bank) or ZNF469 (1:1,000; custom generated by Synbio Technologies). Subsequently, the cells were incubated with Alexa Fluor 488-conjugated anti-mouse or anti-rabbit secondary antibodies (1:500; cat. nos. A-21202 and A-21206, respectively; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 1 h, following by staining with 300 nM 4',6-diamidino-2-phenylindole (DAPI) nuclear stain at room temperature for 10 min. Images were captured using the EVOS M7000 Imaging System, with fluorescence from the DAPI and green fluorescent protein channels taken at the same exposure for comparison.

For analysis of collagen fibril formation, cells were maintained in 24-well plates with the culture medium containing 20 μ M L-ascorbic acid for 8 days. After incubation, cells were fixed and prepared following the method outlined above.

Cleavage under targets and release using nuclease (cut and run) assay. Cut and run assays were performed using the CUT&RUN Assay Kit (cat. no. 86652; CST Biological Reagents Co., Ltd.) according to the manufacturer's instructions. Briefly, 1×10^5 cells were harvested and immobilized on Concanavalin A-coated magnetic beads in binding buffer. Bead-bound cells were then permeabilized and incubated with either normal rabbit IgG antibody as a negative control or anti-ZNF469 antibody. Following washes, protein A-micrococcal nuclease fusion protein was added to induce targeted DNA cleavage. Released DNA fragments were extracted, purified using a spin column kit, and subjected to qPCR analysis. Primers were designed using PrimerQuest based on promoter sequences obtained from the UCSC Genome Browser (<https://genome.ucsc.edu/>). Primer sequences used for qPCR are listed in Table SI.

RNA-seq analysis. Total RNA was extracted from sh1-ZNF469 knockdown cells and the corresponding cells without doxycycline induction. The RNA-seq analysis of the samples was carried out by Beijing Biomarker Technologies Co., Ltd. RNA quality and integrity were assessed using a 2100 Bioanalyzer system (Agilent Technologies, Inc.). RNA libraries were generated using the NEBNext Ultra II RNA Library Prep Kit for Illumina (cat. no. #E7770; New England Biolabs, Inc.) according to the manufacturer's protocol. The concentration of the final library was assessed using Qubit 2.0 (Thermo Fisher Scientific, Inc.) and diluted to a 2 nM loading concentration.

Sequencing was performed on an Illumina NovaSeq 6000 platform (Illumina, Inc.) using paired-end 150-bp reads with NovaSeq X Series25B Reagent Kit (cat. no. 20104706; Illumina, Inc.). Differential gene expression analysis was conducted using DESeq2 (version 1.30.1; <http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html>). Genes with an absolute fold change >1.5 and a false discovery rate (FDR) <0.05 were considered differentially expressed. Gene set enrichment analysis (GSEA; version 4.3.3; Broad Institute, Inc.; <https://www.gsea-msigdb.org/gsea/index.jsp>) was performed on the downregulated genes using a custom gene set database enriched for ECM-related genes to identify enriched Gene Ontology (GO) terms and Reactome pathways. Pathways with an FDR <0.05 were considered significantly enriched. Leading-edge analysis was performed within GSEA to identify core genes contributing to the enriched pathways, which were subsequently validated by RT-qPCR.

Bulk RNA-seq analysis of public datasets. Publicly available bulk RNA-seq datasets were utilized for re-analysis. Specifically, RNA-seq data from hypertrophic scar tissue was obtained from the Gene Expression Omnibus (GEO) database under accession number GSE178411 (unpublished dataset). Additionally, RNA-seq data from keloid tissue were retrieved from the GEO under the following accession numbers: GSE158395 (30), GSE232079 (31), GSE117887 (32), GSE237752 (unpublished dataset), GSE211150 (unpublished dataset), GSE202293 (33), GSE210434 (34) and GSE221382 (35). The Spearman rank correlation coefficient (r) was calculated to assess the correlation between ZNF469 gene expression and the expression of genes associated with ECM.

Single-cell analysis of keloid tissue. Single-cell RNA-seq (scRNA-seq) data from keloid tissue in GEO accession number GSE163973, were reanalyzed as previously described in the original study (36). Cell clustering and annotation were performed to identify skin cell subpopulations, including fibroblast populations. The expression patterns of ZNF469 were then examined across these identified cell clusters. Furthermore, the expression levels of ZNF469 and collagen type I $\alpha 1$ (COL1A1) were specifically analyzed in individual normal scar fibroblasts (NS1, NS2 and NS3) and keloid fibroblasts (KF1, KF2 and KF3). Diffusion Pseudotime analysis was conducted using the Destiny R package in R (version 3.20.0) (37) to infer cellular differentiation trajectories (38).

Statistical analysis. Data are presented as the mean $\pm$ standard deviation and were analyzed using GraphPad Prism software version 10.4.0 (Dotmatics). Differences were analyzed by unpaired Student's t-test or Mann-Whitney U test using a minimum of three replicates from at least two independent experiments. $P < 0.05$ was considered to indicate a statistically significant result.

Results

ZNF469 knockdown impairs dermal fibroblast phenotypes and collagen function. To elucidate the role of ZNF469 in dermal fibroblasts, a loss-of-function experiment using shRNA was

performed. Two distinct shRNA constructs targeting ZNF469 were generated, and successful knockdown was confirmed by RT-qPCR and western blot analysis (Figs. 1A and B, and S1). Importantly, the non-targeting scramble shRNA control, with and without doxycycline treatment, showed no impact on ZNF469 expression, confirming the specificity of ZNF469 knockdown and that doxycycline induction itself does not affect ZNF469 levels (Fig. 1A). Knockdown of ZNF469 significantly impaired dermal fibroblast phenotypes, as evidenced by reduced cell proliferation and migration compared with that of the sh-ZNF469 transduced controls that were not induced with doxycycline (Fig. 1C and D). Furthermore, ZNF469 knockdown attenuated the activated fibroblast phenotype, as demonstrated by a significant reduction in collagen contraction (Fig. 1E). To investigate the impact of ZNF469 on fibrotic and myofibroblast markers, RT-qPCR analysis was performed. The results of this analysis revealed that ZNF469 knockdown specifically decreased the expression of various collagen genes, while other fibrotic and myofibroblast markers, including α -smooth muscle actin (ACTA2) and fibronectin 1 remained unchanged (Fig. 1F). While ZNF469 expression was observed to impact the expression of the basement membrane component-encoding gene COL4A1 in dermal fibroblasts, the transcript levels of COL4A1 were ~16-fold lower than those of COL1A1. This is consistent with the known primary role of fibroblasts in the production of interstitial collagens (39–42). High expression of ACTA2 was also observed in the dermal fibroblasts; this may be attributable to the presence of FBS, which is known to induce ACTA2 and drive fibroblasts toward a myofibroblast-like phenotype (43). Consistent with these findings, IF staining and collagen secretion assays showed that ZNF469 depletion led to a reduction in the intracellular production and extracellular secretion of type I collagen in dermal fibroblasts (Fig. 1G and H). To further examine collagen fibril formation, cells were cultured for an extended period of 8 days prior to IF staining, which revealed diminished fibril accumulation upon ZNF469 depletion (Fig. S2). Furthermore, all observed phenotypes were consistent for both shRNA constructs, suggesting that the phenotypes resulted from the direct effect of ZNF469 knockdown. Collectively, these data suggest that ZNF469 plays a critical role in the regulation of dermal fibroblast function, particularly collagen-associated processes.

ZNF469 depletion disrupts a broad spectrum of ECM-related pathways. To identify the molecular mechanisms underlying the observed phenotypic changes following ZNF469 knockdown, RNA-seq of ZNF469-knockdown and non-doxycycline-induced control dermal fibroblasts was performed. Transcriptome analysis revealed 194 upregulated and 349 downregulated differentially expressed genes (DEGs) in ZNF469-knockdown cells compared with the controls (Fig. 2A and Table SII). GSEA of the downregulated DEGs identified the significant enrichment of GO terms and Reactome pathways associated with collagen processes, including 'complex of collagen trimers', 'collagen trimer' and 'collagen biosynthesis and modifying enzymes', indicating a profound impact of ZNF469 depletion on these pathways. In addition, ECM- and fibroblast-associated pathways were also significantly affected (Figs. 2B–D and S3). Notably,

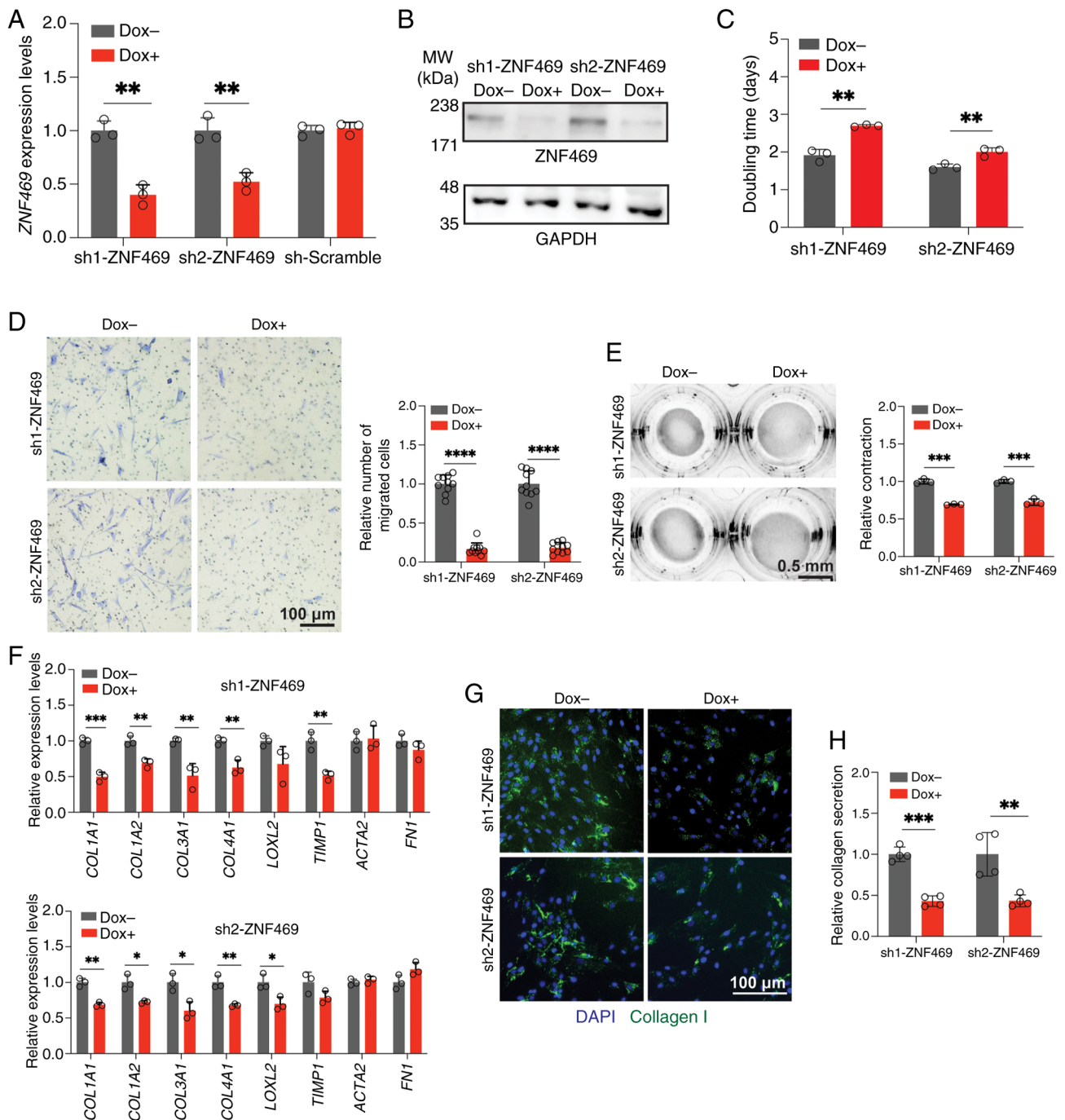


Figure 1. ZNF469 knockdown disrupts key properties of dermal fibroblasts. (A) *ZNF469* mRNA expression in dermal fibroblasts after knockdown using sh1-ZNF469 and sh2-ZNF469 or transduction with sh-Scramble, with and without Dox induction, as determined by RT-qPCR. (B) Assessment of ZNF469 protein levels by western blotting in control and ZNF469-knockdown cells. (C) Doubling time determined by MTT assay during the exponential growth phase of control and ZNF469-knockdown cells. (D) Migration of control and ZNF469-knockdown cells as determined by Transwell assay. (E) Collagen gel contraction in control and ZNF469-depleted cells. (F) Analysis of key fibrotic and myofibroblast markers via RT-qPCR following ZNF469 knockdown. (G) Type I collagen detection by immunofluorescence in ZNF469-knockdown cells compared with control cells. Fluorescent images showing DAPI nuclear staining in blue and type I collagen staining in green were captured under identical exposure settings for comparison. Scale bar, 100 μ m. (H) Collagen secretion levels in ZNF469-knockdown and control cells. The dermal fibroblasts were cultured for 4 days to allow for effective Dox-mediated induction of ZNF469 knockdown prior to all experiments. Data are shown as the mean $\pm$ SD; $n \geq 3$ replicates and ≥ 2 independent experiments. Statistical significance was determined using unpaired Student's t-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ as indicated. ZNF469, zinc finger 469; sh, short hairpin; Dox, doxycycline; RT-qPCR, reverse transcription-quantitative PCR; MW, molecular weight; COL, collagen; LOXL2, lysyl oxidase like 2; TIMP1, TIMP metalloproteinase inhibitor 1; ACTA2, α -smooth muscle actin; FN1, fibronectin 1; DAPI, 4',6-diamidino-2-phenylindole.

several genes encoding ECM components were found to be downregulated upon ZNF469 knockdown, including *COL1A1*, a collagen gene previously identified as a ZNF469 target (14,16,21). Other collagen genes, namely *COL3A1*,

COL5A1, *COL11A1* and *COL15A1*, along with integrin subunit $\alpha 2$ and 6, fibronectin leucine rich transmembrane protein 2 (*FLRT2*), fibrillin 2 (*FBN2*) and osteonectin (*SPARC*), also exhibited reduced expression (Fig. 2E). Furthermore, genes

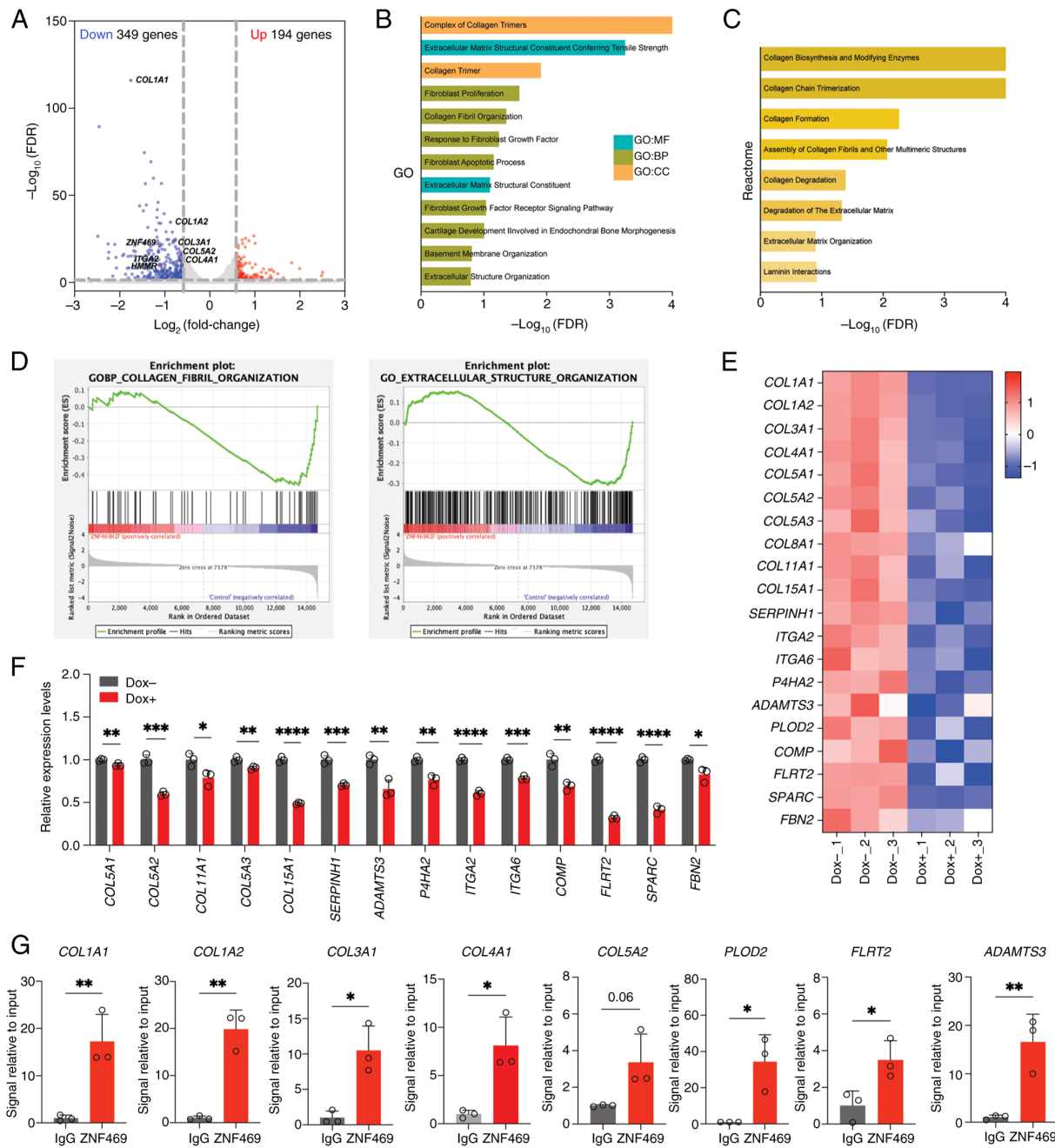


Figure 2. RNA-seq analysis of dermal fibroblasts following ZNF469 knockdown. (A) Volcano plot showing absolute $\log_2$ fold changes and statistical significance, represented as the $-\log_{10}(\text{FDR})$, for gene expression in ZNF469-knockdown cells compared with control cells without Dox induction. DEGs were identified based on an absolute fold change >1.5 and $\text{FDR} < 0.05$. Significantly upregulated and downregulated DEGs are shown as red and blue dots, respectively. (B) GO enrichment analysis of downregulated DEGs in ZNF469-knockdown cells. (C) Reactome pathway analysis of downregulated DEGs in ZNF469-depleted cells. (D) Gene set enrichment analysis plots for representative signaling pathways in ZNF469-knockdown cells. (E) Heat map of representative ECM-related gene expression from the RNA-seq analysis of control and ZNF469-knockdown cells, where red represents upregulated gene expression and blue represents downregulated gene expression, as indicated by a Z-score scale from -1 (blue) to 1 (red). (F) RT-qPCR analysis of representative ECM-related gene expression following ZNF469 knockdown. (G) qPCR analysis of ECM-related gene promoters following the cleavage under targets and release using nuclease assay targeting ZNF469. Data are shown as the mean $\pm$ SD; $n=3$ replicates. Statistical significance was determined using unpaired Student's t-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. RNA-seq, RNA sequencing; ZNF469, zinc finger 469; FDR, false discovery rate; Dox, doxycycline; DEGs, differentially expressed genes; GO, Gene Ontology; MF, molecular function; BP, biological process; CC, Cellular Component; RT-qPCR, reverse transcription-quantitative PCR; ECM, extracellular matrix; COL, collagen; SERPINH1, serpin family H member 1; ADAMTS3, ADAM metalloproteinase with thrombospondin type 1 motif 3; P4HA2, prolyl 4-hydroxylase subunit α 2; ITGA2, integrin α 2; COMP, cartilage oligomeric matrix protein; FLRT2, fibronectin leucine rich transmembrane protein 2; SPARC, osteonectin; FBN2, fibrillin 2; PLOD2, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2.

involved in collagen processing and ECM organization, such as serpin family H member 1, ADAM metalloproteinase with thrombospondin type 1 motif 3 and procollagen-lysine,

2-oxoglutarate 5-dioxygenase 2 (*PLOD2*), were similarly downregulated. In the context of angiogenesis, certain markers were found to be affected by ZNF469 knockdown, including

vascular endothelial growth factor A, fibroblast growth factor 2 and angiopoietin 1. By contrast, other angiogenic markers such as platelet-derived growth factors D and C, interleukin-6 (*IL6*) and *IL33* remained unchanged (Fig. S4). While certain angiogenesis markers showed some degree of alteration upon ZNF469 depletion, none met the study criteria for differential expression. Further studies are necessary to elucidate the underlying mechanisms and the broader implications of ZNF469 in angiogenic regulation.

To validate the RNA-seq findings, RT-qPCR was performed, which confirmed the reduced expression of ECM-related genes following ZNF469 knockdown (Fig. 2F). In addition, the results of the CUT&RUN assay, a technique used for the study of chromatin-associated proteins, such as transcription factors and histone modifications, demonstrated direct binding of ZNF469 to the promoters of ECM-related genes, suggesting that ZNF469 may transcriptionally regulate their expression (Fig. 2G). These results indicate that ZNF469 is essential for the maintenance of ECM homeostasis in dermal fibroblasts.

ZNF469 is upregulated and correlates with ECM-related genes in pathological skin scarring. To evaluate the clinical importance of ZNF469 in fibrotic skin disorders, publicly available bulk RNA-seq datasets derived from hypertrophic and keloid scar tissues were reanalyzed. Hypertrophic scars are typically restricted to the original wound area, whereas keloids are more aggressive, therapy-resistant lesions that extend beyond the wound boundaries. Moreover, research has demonstrated the induction of both *COL1A1* and *COL3A1* in keloid scars, with *COL1A1* being induced to a much greater extent (44). Analysis of hypertrophic scar tissue in the GSE178411 dataset revealed a significant upregulation of the expression of both ZNF469 and ECM-related genes compared with that in normal skin tissue (Fig. 3A). Furthermore, ZNF469 expression levels were strongly correlated with the expression levels of ECM-related genes in hypertrophic scars, with the exception of *FLRT2*, which demonstrated a moderate association (Fig. 3B). Consistent with these findings, the analysis of the keloid tissue datasets GSE158395, GSE232079, GSE117887, GSE237752, GSE211150, GSE202293, GSE210434 and GSE221382, also demonstrated a significant increase in ZNF469 and ECM-related gene expression in keloid tissue compared with normal skin (Fig. 3C). ZNF469 expression correlated strongly with *COL1A1* and *COL1A2* expression and moderately with *COL3A1*, *COL5A1*, *COL11A1* and *SPARC* expression, while fair correlations were noted with *COL4A1*, cartilage oligomeric matrix protein (*COMP*), *FBN2* and *PLOD2* expression in keloid tissue (Fig. 3D). However, the magnitude of upregulation and the strength of the correlations in keloid samples appeared to be lower compared with those in hypertrophic scars, potentially reflecting inherent variability across the diverse keloid datasets. However, collectively, these results suggest that ZNF469 plays a crucial role in the pathogenesis of pathological skin scarring.

Single-cell RNA-seq analysis identifies ZNF469 as a regulator of mesenchymal fibroblasts in keloid scarring. To further investigate the role of ZNF469 in dermal fibroblasts, the publicly available GSE163973 scRNA-seq dataset from

normal and keloid scars was reanalyzed, focusing specifically on ZNF469 expression (36). The analysis revealed the presence of diverse cell populations within the skin tissue, including keratinocytes, fibroblasts, endothelial cells and melanocytes, with ZNF469 predominantly expressed in fibroblasts (Figs. 4A, S5 and S6). Subclustering of the fibroblast population identified four distinct subpopulations, namely mesenchymal, pro-inflammatory, secretory reticular and secretory papillary fibroblasts. Notably, the mesenchymal fibroblast subpopulation, implicated in keloid scar formation, was significantly enriched in keloid tissue compared with normal tissue (45,46). Furthermore, ZNF469 expression in mesenchymal fibroblasts from keloid tissue was significantly higher compared with that in mesenchymal fibroblasts from normal tissue (Figs. 4B and S6). Consistent with these findings, individual keloid fibroblasts exhibited higher expression levels of ZNF469 and ECM-related genes, such as *COL1A1*, compared with those in normal fibroblasts (Fig. 4C). Moreover, positive correlations between ZNF469 and ECM-related gene expression levels were observed within the single-cell data (Fig. 4D), corroborating the bulk RNA-seq findings. Collectively, these results suggest that ZNF469 plays a critical role in the regulation of mesenchymal fibroblast activity, potentially contributing to excessive collagen production and fibrosis in keloid tissue.

Building upon the observed association between ZNF469 expression and mesenchymal fibroblasts in keloid and normal scar tissues, the role of ZNF469 in this specific fibroblast population was further investigated using dermal fibroblast cells. Consistent with the RNA-seq data, ZNF469 knockdown was found to result in a significant reduction of mesenchymal marker expression (Fig. 5A). This suppression was confirmed by RT-qPCR analysis (Fig. 5B). Notably, while the RNA-seq analysis did not reveal a significant change in *COL12A1* expression upon ZNF469 knockdown, RT-qPCR analysis indicated a significant downregulation, potentially reflecting differences in detection sensitivity. Notably, keloid scarring shares similarities with cartilage formation, a process dependent on several mesenchymal genes (36,47). The present study revealed that ZNF469 knockdown significantly impacted pathways associated with cartilage development and bone morphogenesis (Fig. S7). CUT&RUN assays demonstrated that ZNF469 binds to the promoters of mesenchymal genes, supporting its role in the transcriptional regulation of these genes (Fig. 5C). Furthermore, pseudotime analysis of mesenchymal fibroblast subpopulations, derived from the reanalysis of scRNA-seq data, revealed two distinct populations: Population 0 and population 3 (Fig. 5D). Diffusion mapping of gene expression showed that ZNF469 and the mesenchymal marker periostin (*POSTN*) were highly expressed in population 3, supporting the hypothesis that ZNF469 controls mesenchymal gene expression (Fig. 5D). Integrating these pseudotime analysis findings with previously published RNA velocity data, which identified four differentiation trajectories of mesenchymal fibroblasts, suggests that population 3 represents an early, more mesenchymal-like fibroblast state (36). This population may gradually differentiate into the less mesenchymal-like population 0 over time. As a result, it is suggested that these mesenchymal-like fibroblasts might represent a pioneer population responsible for triggering fibrotic processes. Overall,

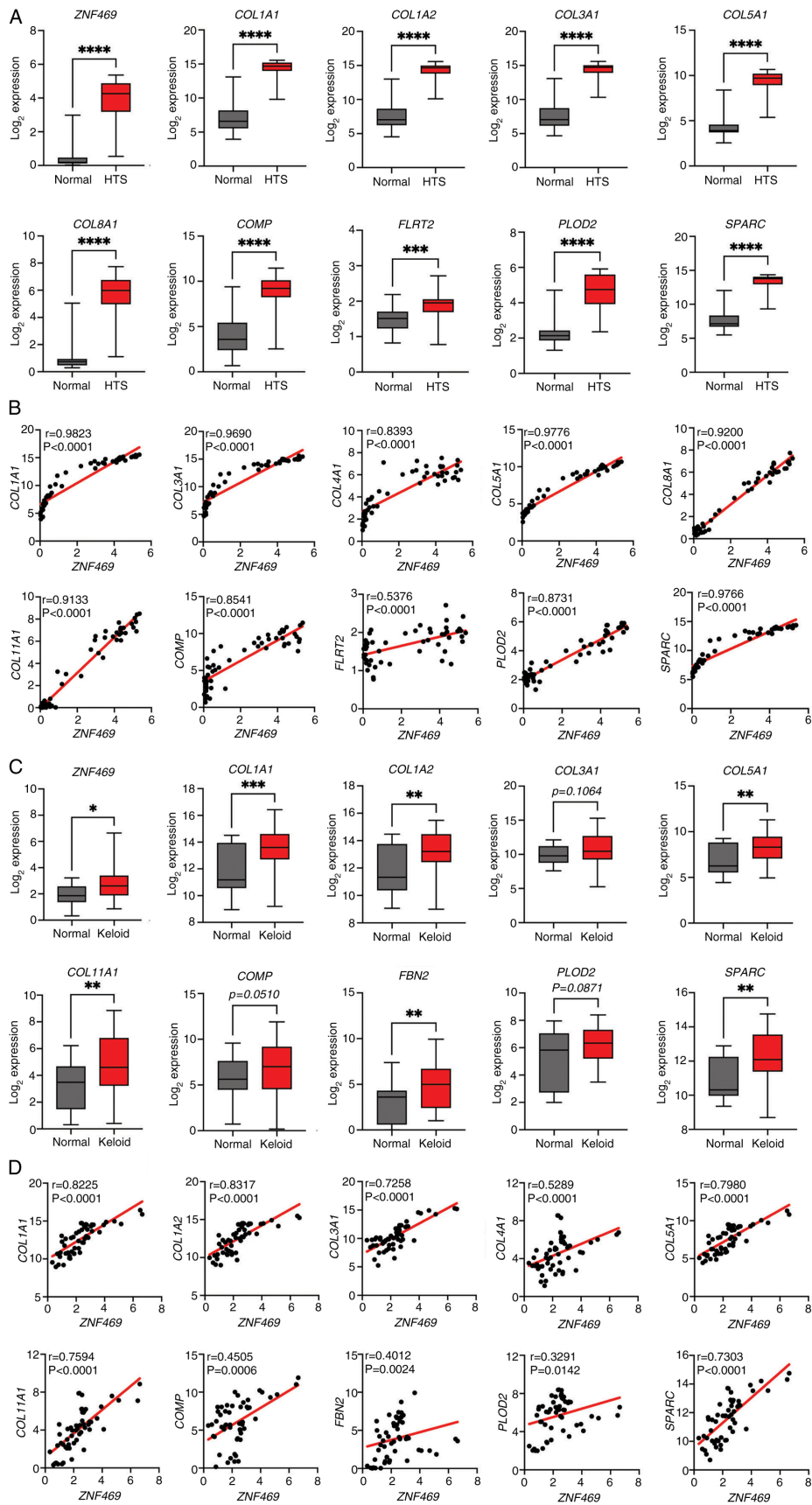


Figure 3. ZNF469 upregulation correlates with increased ECM gene expression in hypertrophic and keloid scars. (A) Re-analysis of RNA-seq data from the GSE178411 dataset presented as box-and-whisker plots, illustrating ZNF469 and ECM-related gene expression levels in normal (n=24) and hypertrophic (n=28) scar tissues. (B) Spearman correlation plots assessing the correlation between ZNF469 expression and ECM-related gene expression in normal and hypertrophic scar samples. (C) Re-analysis of RNA-seq data from several Gene Expression Omnibus datasets showing ZNF469 and ECM gene expression levels in normal (n=26) and keloid (n=29) scar tissues. (D) Spearman's correlation plots for ZNF469 and ECM-related gene expression in normal and keloid scar samples. (A) and (C) were analyzed using Mann-Whitney U test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. ZNF469, zinc finger 469; ECM, extracellular matrix; RNA-seq, RNA sequencing; HTS, hypertrophic scar.

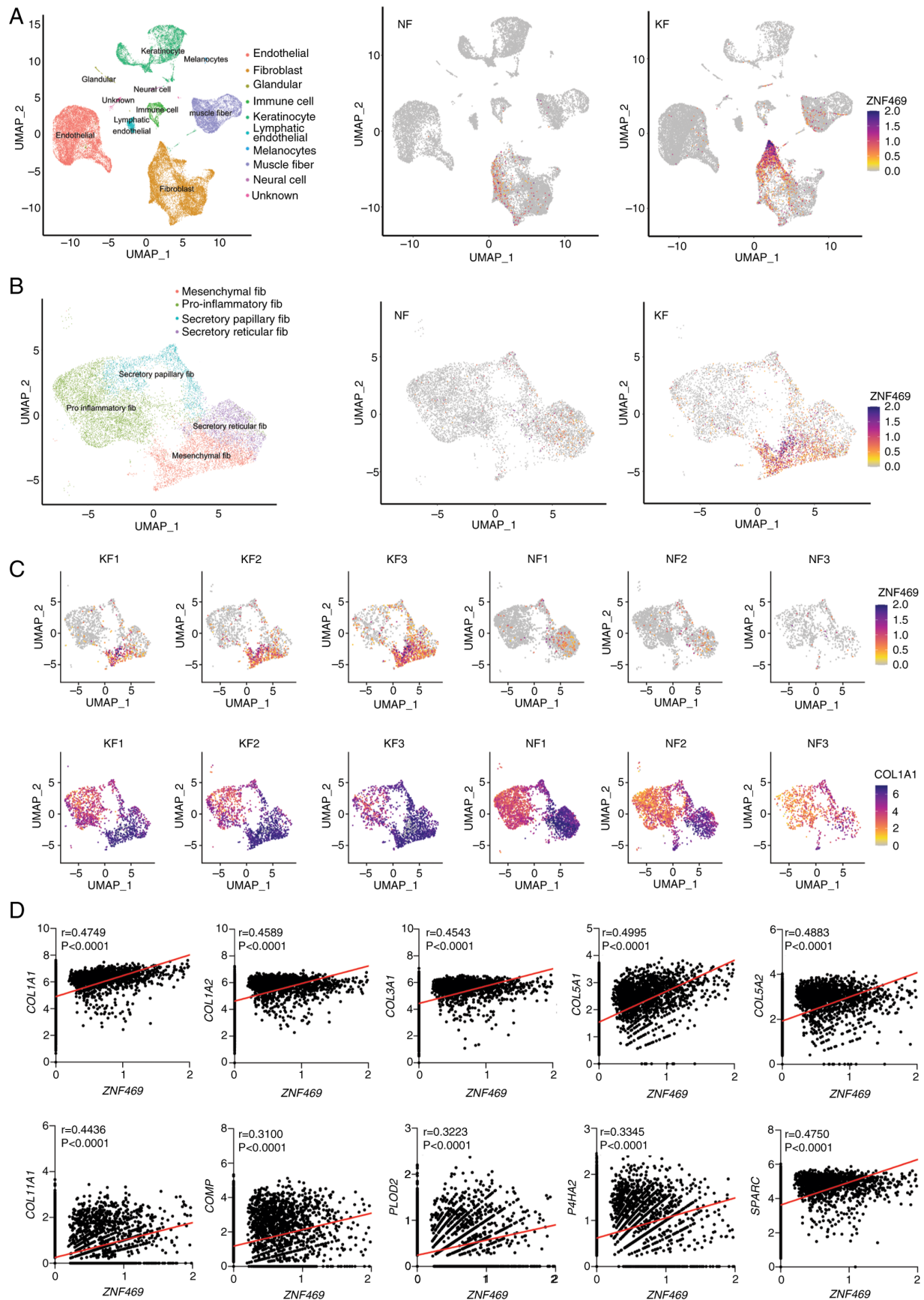


Figure 4. Single-cell RNA-sequencing analysis of ZNF469 expression in normal and keloid scars. Dataset GSE163973 was utilized for the analysis. (A) UMAP analysis of skin cell populations and the distribution of ZNF469 expression in normal and keloid scars. (B) UMAP plot depicting fibroblast subpopulations and ZNF469 expression across NFs and KFs. (C) Expression patterns of ZNF469 and COL1A1 in fibroblasts from the skin samples of six individuals (NF1-3 and KF 1-3) are shown with expression levels indicated by colors ranging from grey to purple on a scale of 0 to 2, representing low to high expression. (D) Spearman's correlation plots for ZNF469 expression and extracellular matrix-related gene levels in normal and keloid scar samples. ZNF469, zinc finger 469; UMAP, Uniform Manifold Approximation and Projection; KF, keloid scar fibroblasts; NF, normal scar fibroblasts; COL1A1, collagen type I α 1.

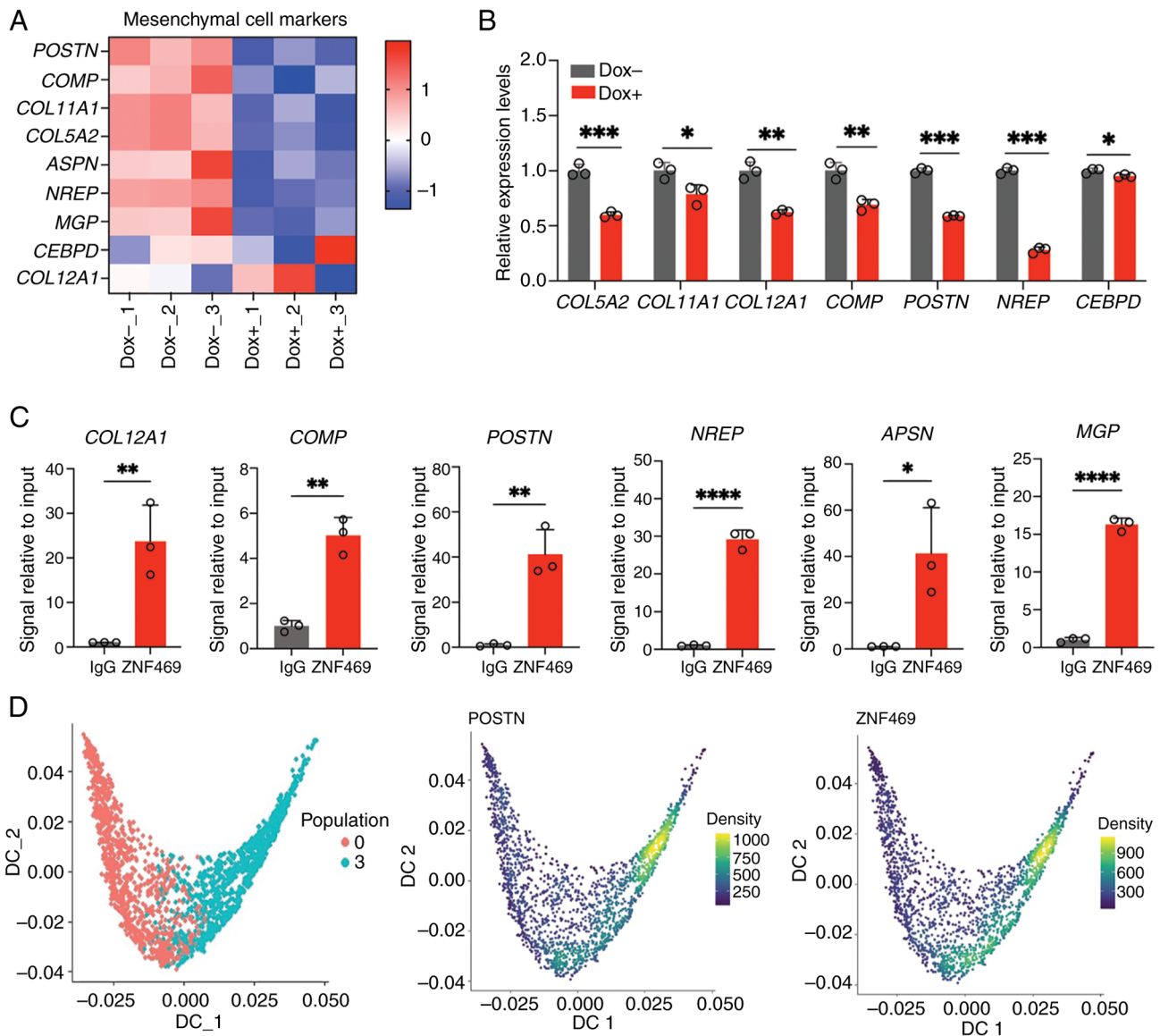


Figure 5. ZNF469 regulates a mesenchymal fibroblast subpopulation. (A) Mesenchymal marker gene expression profiles in ZNF469-knockdown cells compared with control cells without Dox induction based on RNA-sequencing data, where red represents upregulated gene expression and blue represents downregulated gene expression, according to a Z-score scale from -1 (blue) to 1 (red). (B) RT-qPCR evaluation of mesenchymal markers after ZNF469 knockdown. (C) qPCR analysis of mesenchymal gene promoters following the cleavage under targets and release using nuclease assay targeting ZNF469. (D) Pseudotime analysis of mesenchymal fibroblasts and diffusion maps of *POSTN* and *ZNF469* expression. Statistical significance was determined using unpaired Student's t-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. ZNF469, zinc finger 469; Dox, doxycycline; RT-qPCR, reverse transcription-quantitative PCR; POSTN, periostin; COL, collagen; COMP, cartilage oligomeric matrix protein; NREP, neuronal regeneration related protein; CEBPD, CCAATenhancer binding protein δ ; ASPN, asporin; MGP, matrix Gla protein.

these findings indicate that ZNF469 plays a critical role in the regulation of mesenchymal fibroblast subpopulations, potentially contributing to the initiation of fibrosis in skin disorders.

ZNF469 knockdown suppresses collagen production in keloid fibroblasts. To evaluate the therapeutic potential of ZNF469 as a target for keloid fibrosis, a loss-of-function experiment was performed using a keloid fibroblast cell line. Successful ZNF469 knockdown was confirmed by RT-qPCR and western blot analysis (Figs. 6A and B, and S1). Consistent with the observations in dermal fibroblasts, ZNF469 depletion significantly impaired keloid fibroblast properties, including cell proliferation and migration (Fig. 6C and D). Furthermore,

ZNF469 knockdown reduced collagen contraction, indicating a reduction in activated fibroblast activity (Fig. 6E). The quantitative analysis of ECM-related and mesenchymal fibroblast markers, such as *POSTN*, *COMP* and *COL11A1* (36,48-50), by RT-qPCR revealed a significant reduction in their expression upon ZNF469 knockdown (Fig. 6F). These results suggest that ZNF469 plays a crucial role in the regulation of mesenchymal fibroblast behavior and ECM composition in keloid fibroblasts. Moreover, IF and collagen secretion assays demonstrated a significant reduction in type I collagen production and secretion following ZNF469 knockdown (Fig. 6G and H). Collectively, these findings indicate that the knockdown of ZNF469 expression effectively reversed ECM-related abnormalities in keloid fibroblasts, highlighting its potential as a therapeutic target for keloid fibrosis.

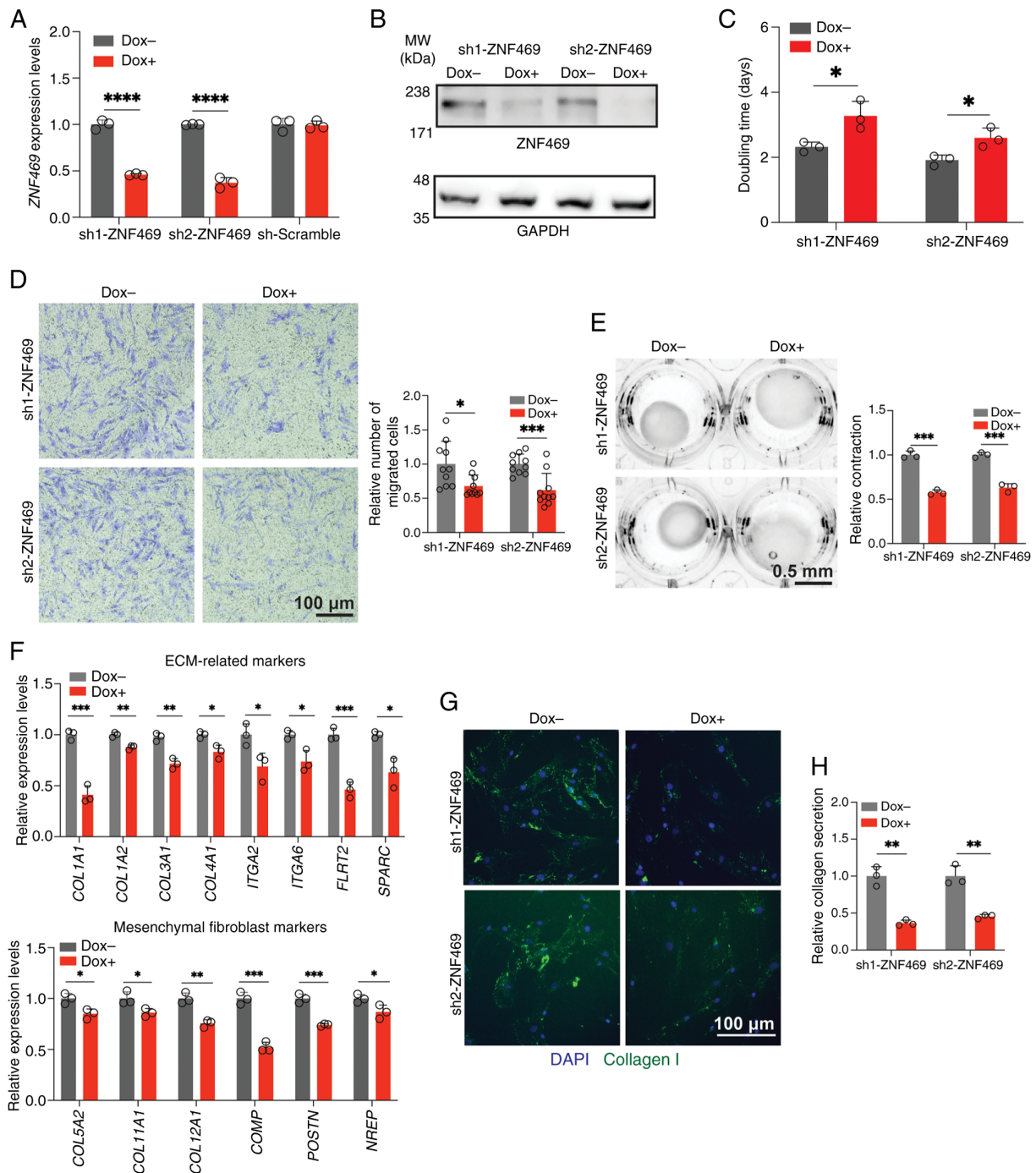


Figure 6. Knockdown of ZNF469 changes the characteristics of keloid fibroblasts. (A) RT-qPCR analysis of ZNF469 expression in keloid fibroblasts following knockdown using sh1-ZNF469 and sh2-ZNF469 or transduction with sh-Scramble, with and without Dox, as determined by RT-qPCR. (B) Western blot assay of ZNF469 expression in control and ZNF469-knockdown cells. (C) Comparison of doubling times during the exponential growth phase between control and ZNF469-depleted cells, measured by MTT assay. (D) Transwell migration assay of control and ZNF469-depleted cells. (E) Collagen gel contraction analysis in control and ZNF469-knockdown cells. (F) Evaluation of key fibrotic and mesenchymal markers via RT-qPCR analysis following ZNF469 knockdown. (G) Immunofluorescence of type I collagen in control and ZNF469-knockdown cells. Fluorescent images were acquired using consistent exposure settings for comparison. Scale bar, 100 μ m. (H) Secreted collagen levels of control and ZNF469-knockdown cells. Keloid fibroblasts were cultured for 4 days to allow for the effective Dox-mediated induction of ZNF469 knockdown prior to all experiments. Data are presented as the mean $\pm$ SD of $n \geq 3$ replicates from 2 independent experiments. Statistical significance was evaluated using unpaired Student's t-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. ZNF469, zinc finger 469; sh, short hairpin; Dox, doxycycline; RT-qPCR, reverse transcription-quantitative PCR; MW, molecular weight; ECM, extracellular matrix; ITGA2/6, integrin α 2/6; FLRT2, fibronectin leucine rich transmembrane protein 2; SPARC, osteonectin; COMP, cartilage oligomeric matrix protein; POSTN, periostin; NREP, neuronal regeneration related protein; DAPI, 4',6-diamidino-2-phenylindole.

Discussions

Fibroblasts are essential for ECM production and wound healing; however, their dysregulation can result in fibrosis,

such as keloid formation (51-53). The ZNF469 mutation, initially discovered in patients with BCS, was later explored in zebrafish and mouse models of BCS, which revealed its role as a profibrotic factor (18,19,54,55). However, its role in common

fibrotic organs, particularly the skin, is largely unexplored. The present study demonstrates that ZNF469 knockdown in dermal fibroblasts significantly disrupts critical fibroblast functions, including migration, proliferation and contraction, and also impairs collagen-related processes, such as collagen secretion. Comprehensive RNA-seq analysis revealed that the modulation of ZNF469 expression profoundly impacts ECM-associated pathways, particularly those regulating collagen biosynthesis. Notably, bulk RNA-seq data from hypertrophic and keloid scars revealed a concurrent upregulation and positive correlation between ZNF469 and ECM-related gene expression levels. Furthermore, single-cell RNA-seq reanalysis highlighted the predominant expression of ZNF469 in fibroblasts, particularly mesenchymal fibroblasts within keloid tissues, underscoring its potential involvement in skin fibrosis development via these cells. Crucially, silencing ZNF469 in keloid fibroblast models reduced excessive fibroblast activity and collagen production, indicating its possible use as a therapeutic target. Collectively, the present study provides the first comprehensive evidence that ZNF469 is a critical ECM regulator in skin fibroblasts, suggesting its promise as a marker and therapeutic target for skin fibrosis.

The present study corroborates the critical role of mesenchymal fibroblasts in fibrosis, further establishing ZNF469 as a key regulator within this fibroblast subpopulation. This aligns with prior research recognizing the influence of ZNF469 on mesenchymal fibroblasts and their important contribution to keloid development through complex cellular interactions (36). Notably, the pseudotime analysis performed in the present study and RNA velocity analyses from a previous study suggest that ZNF469, among other mesenchymal markers, governs an early mesenchymal fibroblast state that progresses into mature ECM-producing fibroblasts (36). Extending these insights, a study on diabetic foot ulcers indicates that mesenchymal fibroblasts may ultimately differentiate into myofibroblasts, driving sustained fibrotic activity (56). Consistent with this observation, skin fibrosis conditions such as scleroderma are characterized by an increased abundance of mesenchymal fibroblasts, in contrast with aging, where their proportion decreases (36,56,57). Collectively, the present study reveals that ZNF469 is a specific regulator of mesenchymal fibroblasts in skin fibrosis, a role that was previously unrecognized. This finding establishes ZNF469 upregulation as a key driver in the pathogenesis of skin fibrosis, distinct from its broader contributions to ECM regulation. These results necessitate further investigation of the intricate mechanisms by which ZNF469 modulates mesenchymal fibroblast activity across diverse skin conditions, including aging, which contrasts with fibrosis in its fibroblast dynamics. Notably, the single-cell data suggest that while *COL1A1* expression occurs independently of ZNF469 in some fibroblasts, ZNF469 appears to function cell-autonomously to promote high-level *COL1A1* expression within a specific, collagen-producing fibroblast subpopulation in keloids.

Beyond its established role in ECM regulation, the findings of the present study suggest ZNF469 may also play a role in skeletal development. The RNA-seq data revealed DEG enrichment within the GO biological process 'Cartilage development involved in endochondral bone morphogenesis' pathway, in addition to other pathways associated with bone

and cartilage formation. This observation aligns with previous research demonstrating a strong association of genes enriched in keloid and systemic sclerosis datasets with proteins integral to bone and cartilage (47). Furthermore, several mesenchymal genes, including *COL5A2*, *COL11A1* and *COMP*, which were identified as ZNF469 targets, are known to be upregulated in bone- and cartilage-related diseases (58-60). Notably, PRDM5, a known regulator of collagen that is functionally similar to ZNF469, is highly expressed in bone tissue and significantly impacts osteogenesis when depleted (17,61). Collectively, these results strongly indicate a previously unknown role for ZNF469 in bone and cartilage biology, warranting further investigation of its precise mechanisms within these pathways.

The therapeutic landscape for skin fibrosis disorders is continually advancing, as researchers seek to maximize efficacy while minimizing adverse effects, thereby improving patient quality of life. Current treatment modalities range from traditional surgical interventions, radiotherapy and intralesional injections targeting fibroblast pathways (62) to innovative approaches including small molecule inhibitors of fibrosis pathways, adipose stem cell therapy and pulsed electric field therapy (63-65). The identification of ZNF469 as a critical regulator of fibrosis in skin fibroblasts, particularly in keloid models, opens new avenues for targeted therapies. A primary hurdle in the translation of these findings into clinical applications is the effective delivery of therapeutic agents across the epidermal barrier. However, emerging technologies, such as the transdermal delivery of small interfering RNA using lipid nanocarriers and microneedle patches offer promising solutions that could be adapted to enhance shRNA delivery to the dermal layer (66,67). In addition to delivery challenges, the selection of optimal shRNA candidates with minimal off-target effects for ZNF469 knockdown remains a crucial consideration for future therapeutic development.

The present study is limited by the absence of gain-of-function analyses, which restricts a comprehensive understanding of the biological roles of ZNF469 beyond loss-of-function effects. Achieving the efficient overexpression of a large ZNF469 fragment in dermal fibroblasts has been challenging, and attempts to establish a CRISPR activation system yielded unsatisfactory results. Further optimization of ZNF469 overexpression methods is necessary to fully elucidate its function in dermal fibroblasts and other tissues. In addition, while type I collagen was successfully validated at the protein level, the absence of similar validation for other key ECM genes is another limitation of the study. The protein-level validation of these genes will be prioritized in future research to provide stronger support for the transcriptomic findings. Furthermore, the lack of *in vivo* analysis in the present study is another limitation. Due to the lack of commercially available ZNF469-mutant mice, the development of custom-generated models is necessary, which is a resource-intensive endeavor. However, the establishment ZNF469-deficient mice should provide valuable insights into the biological functions of this gene. It is also important to address the observation that baseline ZNF469 protein levels in the keloid and normal fibroblast cell lines appeared similar in the western blot assays, which may be influenced by donor-specific factors such as age, ethnicity

and sex. Future studies with a larger number of cell lines from diverse donors are needed to confirm these findings and clarify the association between ZNF469 expression and keloid pathogenesis.

In summary, the present study establishes ZNF469 as a critical regulator of ECM production in skin fibroblasts, which may drive fibrosis via a specific mesenchymal fibroblast subpopulation. These findings illuminate a promising avenue for the development of targeted therapeutic interventions aimed at modulating ZNF469 expression in fibrotic skin disorders.

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Availability of data and materials

The RNA-seq data sets generated in the present study may be found in the National Center for Biotechnology Information Gene Expression Omnibus repository under accession number GSE290035. All other data generated in the present study may be requested from the corresponding author.

Authors' contributions

CA conceived, designed and supervised the study. DC performed most of the experiments and analyzed the data with assistance from TB. AN contributed to the bioinformatic analysis. DC and CA wrote the manuscript and all authors edited the manuscript. DC and CA confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Use of artificial intelligence tools

During the preparation of this work, Gemini, an artificial intelligence tool, was used to improve the readability and language of the manuscript. Subsequently, the authors revised and edited the content produced by the artificial intelligence tool as necessary, taking full responsibility for the ultimate content of the present manuscript.

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