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Red light promotes dermis-epidermis remodeling via TGFβ and AKT-mediated collagen dynamics in naturally aging mice

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ABSTRACT

Red light therapy is a clinically validated, noninvasive approach for improving skin structure and stimulating collagen renewal. However, the molecular mechanisms by which light therapy reverses collagen-related skin degeneration remain unclear. Using a natural aging mouse model, this study investigated the effects of red light therapy on skin structure and regeneration. Unlike other wavelengths, red light rapidly increased dermal thickness and stimulated epidermal renewal by enhancing collagen synthesis in dermal fibroblasts and activating collagen/integrin-induced proliferation and differentiation of epidermal keratinocytes, resulting in significant improvements in skin morphology. Mechanistically, red light increased endogenous TGFβ expression in fibroblasts, which up-regulated type I collagen mRNA and protein expression via activation of SMAD2/3/4 nuclear translocation. Simultaneously, red light elevated intracellular cAMP, triggering AKT activation that inhibited matrix metalloproteinase expression via the NRF2/HO-1-dependent pathway, thereby reducing collagen degradation. The accumulation of type I collagen in dermal fibroblasts stimulated integrin signaling, promoting epidermal keratinocyte proliferation and differentiation. Red light-induced AKT activation also enhanced fibroblast proliferation, further amplifying collagen production and collagen-mediated epidermal renewal. These findings elucidate the mechanisms by which red light stimulates endogenous TGFβ and AKT signaling to regulate type I collagen production, driving coordinated dermis-epidermis

remodeling. This pathway represents a potential therapeutic target for the prevention and treatment of age-related dermal degeneration.

Keywords: Red light; Dermal fibroblasts; Epidermal keratinocytes; Type I collagen; Skin degeneration

INTRODUCTION

The growing demand for safe and noninvasive therapies to counteract age-related skin degeneration has spurred intensive research into light-based interventions, particularly red light therapy, which has demonstrated clinical efficacy in promoting collagen synthesis and improving skin texture (Garza et al., 2023; Lee et al., 2015). However, despite its therapeutic potential, the molecular mechanisms underlying these effects remain poorly understood, limiting its translational refinement and broader clinical utility. Addressing this gap is vital for developing targeted strategies to optimize treatment outcomes and expand its dermatological applications (Cohen et al., 2023; Mineroff et al., 2024).

Skin degeneration is characterized by dermal thinning, loss of elasticity, surface roughness, dullness, reduced resilience, and impaired barrier function—manifestations that not only affect appearance but also predispose skin to bruising, infections, delayed wound healing, and chronic dermatological conditions such as xerosis, pruritus, and eczematous dermatitis (Shim, 2019; Yang et al., 2023). These age-related alterations largely reflect a decline in epidermal renewal driven

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by reduced keratinocyte proliferation and differentiation (Goruppi et al., 2023), and a marked depletion of dermal type I collagen, a major component of the skin extracellular matrix (Quan et al., 2021, 2023). Of particular relevance, $\alpha 2\beta 1$ integrin, a collagen receptor highly expressed on keratinocytes (Zeltz & Gullberg, 2016), has been shown to regulate epidermal proliferation and differentiation in response to type I collagen (Bozó et al., 2020; Carley et al., 2021). Hence, restoring keratinocyte self-renewal and reversing collagen deficiency through induction of its synthesis may represent an effective strategy for preventing and treating clinical manifestations of skin degeneration.

In this study, red light was applied to 18–20-month-old mice and cultured dermal fibroblasts to investigate its regenerative potential in aged skin. Red light exposure led to a pronounced thickening of the dermis, attributed to accumulation of type I collagen, and stimulated epidermal renewal via collagen-integrin signaling, which promoted keratinocyte proliferation and differentiation. These regenerative effects were abolished by pharmacological inhibition or genetic silencing of AKT and TGF β receptor 1 (TGF β R1), indicating that both pathways are essential mediators of the red light response. Integrated *in vivo* and *in vitro* analyses substantiated that activation of AKT and TGF β is critical for orchestrating red light-induced remodeling of the dermis and epidermis in aged skin. These findings advance fundamental understanding of how red light modulates the molecular pathways underlying age-related skin degeneration and provide a mechanistic framework to guide the assessment and clinical application of phototherapy in aging skin.

MATERIALS AND METHODS

Mice

C57BL/6 mice were purchased from the Guangdong Medical Laboratory Animal Center (Guangzhou, China). The young group consisted of 2-month-old mice, while the aged group included mice aged 18–20 months. All animals were housed under identical living conditions at a constant temperature ($23\pm 1^\circ\text{C}$), relative humidity (50%–60%), and a 12-h light/dark cycle (lights on from 0700h to 1900h) with free access to food and water. To inhibit AKT, mice received intraperitoneal (i.p.) injections of API-2 (HY-15457, MCE, USA) at a dose of 2 mg/kg every 48 h. To inhibit TGF β , mice received i.p. injections of SB431542 (HY-10431, MCE, USA), a TGF β R1 inhibitor, at a dose of 10 mg/kg every 48 h. To inhibit cAMP, mice received i.p. injections of SQ22536 (HY-100396, MCE, USA), an adenylate cyclase (AC) inhibitor, at a dose of 10 mg/kg every 48 h. To inhibit HO-1, mice received i.p. injections of HO-1i (HY-111798A, MCE, USA) at a dose of 1.357 mg/kg every 48 h. To inhibit intracellular free Ca^{2+} , mice received i.p. injections of diltiazem (HY-B0632, MCE, USA) at a dose of 20 mg/kg every 48 h. To inhibit reactive oxygen species (ROS), mice received daily topical application of 100 mg/mL ascorbic acid (HY-B0166, MCE, USA), applied to the skin 30 min after red light exposure. Reagent details are provided in Supplementary Table S1. All procedures were approved by the Institutional Animal Care and Use Committee of South China Normal University (approval number: SCNU-BIP-2021-029).

Light treatments

Light treatment was conducted following the experimental procedures outlined in our previous study (Chang et al., 2021).

For the *in vivo* experiments, the dorsal skin of mice was shaved to ensure unobstructed light penetration. Mice were maintained in a fully dark or dimly lit environment to minimize potential interference from external light sources. Subsequently, red light was delivered using a semiconductor laser system for 28 consecutive days. The energy density at the skin surface was 8 J/cm², administered over a 20 min session at a power density of 6.67 mW/cm². The precise parameters employed for light treatment in the cellular and murine experiments are detailed in Supplementary Tables S2, S3.

Skin elasticity and transepidermal water loss (TEWL) analyses

Skin elasticity and TEWL were assessed on days 0 and 28 following red light exposure using the TM210 and Cutometer Dual MPA580 devices (Courage and Khazaka Electronic GmbH, Germany). Skin elasticity was measured by applying negative pressure to the skin and measuring the extent of skin deformation. The elasticity value was calculated as the ratio of U_a/U_f , where U_a represents the final retraction and U_f the final deformation. Mice were anesthetized with an intraperitoneal injection of chloral hydrate prior to testing. All measurements were performed in a closed chamber, maintaining a temperature range of 20–22°C and relative humidity of 46%–50%.

Isolation and culture of mouse skin fibroblasts (MSFs) and embryonic fibroblasts (MEFs)

MSFs and MEFs were isolated from the dorsal skin tissue of 6–8-month-old and neonatal C57BL/6 mice within 24 h, respectively. Mice were sacrificed, and excised skin samples were washed twice with phosphate-buffered saline (PBS) and incubated in Dispase II (1 U/mL) at 37°C for 60 min in a 10 cm culture dish. Subsequently, the dermis was manually separated from the epidermis, minced into small pieces, and incubated in 0.2% type I collagenase (YEASEN, China) in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) (Gibco, USA) for 60 min at 37°C, followed by DMEM containing 10% fetal bovine serum (FBS) to stop the digestion process. Cells were filtered through a 40 μm cell strainer and centrifuged at 710 $\times g$ for 8 min at 4°C. The harvested cells were then cultured in DMEM/F12 supplemented with 10% FBS, 100 units/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin for 6–7 days. Non-adherent cells were removed, and the remaining adherent population was designated as dermal fibroblasts. Fibroblasts at passages 2–3 were used for subsequent experiments.

RNA extraction and sequencing

All RNA sample processing, including RNA extraction, cDNA library generation, and RNA sequencing (RNA-seq), was conducted by Banian Medical Technology (Guangzhou) Co., Ltd. Initially, total RNA was extracted from mouse skin using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and verified by RNase-free agarose gel electrophoresis. Subsequently, mRNA was enriched from the skin RNA using Oligo(dT) beads, then fragmented into short pieces with fragmentation buffer and reverse transcribed into cDNA using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB #7530, New England Biolabs, USA). The resulting double-stranded cDNA fragments were purified, end-repaired, A-

tailed, and ligated to Illumina sequencing adapters. The ligation products were purified using AMPure XP Beads (1.0×), size-selected using agarose gel electrophoresis and amplified by polymerase chain reaction (PCR). Finally, the cDNA library was sequenced using the Illumina NovaSeq 6000 platform (USA).

Raw sequencing reads were filtered using fastp (v.0.18.0) to remove adapters and low-quality bases for subsequent assembly and analysis. Transcript abundance was quantified using RSEM, and expression levels were reported as fragments per kilobase of transcript per million mapped reads (FPKM). RNA-seq data visualization was performed using R (v.4.3.1). Differential expression analysis was conducted using DESeq2 (v.1.40.2), and visualization was performed with ggplot2 (v.3.4.2). Genes with a fold change (FC) greater than 2.0 and a false discovery rate (FDR)-adjusted $P < 0.05$ were identified as significantly differentially expressed genes (DEGs) (Supplementary Table S4).

Adeno-associated virus-mediated gene transfer

Knockdown of AKT and TGF β R1 in mouse skin was achieved via subcutaneous delivery of an adeno-associated virus (AAV) that selectively expresses AKT/TGF β R1-shRNA with EGFP (AAV-shAKT/TGF β R1-EGFP). The recombinant plasmids, AAV-GP-1 (pAAV-U6-EGFP), were then packaged into AAV DJ viruses alongside an AAV helper plasmid (pAAV) and purified by ultrafiltration. These procedures were carried out by GenePharma (Suzhou, China). The titers of the recombinant AAV supernatants were determined by quantitative PCR, yielding 2.07×10^{13} viral genomes (VG)/mL for AAV-shAKT/TGF β R1-EGFP and 1.23×10^{13} VG/mL for AAV-shControl-EGFP. The sequences for the scrambled shAKT, shTGF β R1, and shControl were 5'-CCCAGAAC AATTAGATTTCATGTAGA-3', 5'-GATGGTCTTTGCTTTGTCT-3', and 5'-TTCTCCGAACGTGTACAGT-3', respectively.

The recombinant AAV vectors were injected subcutaneously at a dose exceeding 1.23×10^{12} VG/mouse. Two weeks post-injection, light treatment was performed continuously for 28 days, after which skin samples from the mice were collected for testing and analysis. A minimum of four mice per group was used for all experiments, as defined by genotype.

Western blot analysis

Skin tissue and cultured cells were lysed on ice for 40–60 min using lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 1% Triton X-100, 50 mmol/L NaF, 1 mmol/L NaVO₄, 0.1% SDS, and 100 μ g/mL PMSF, supplemented with a protease inhibitor cocktail). Lysates were centrifuged at 13 500 $\times g$ for 20 min at 4°C and quantified using the Bradford assay. Proteins were separated using 8%–15% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). After blocking, the membranes were incubated overnight at 4°C with gentle shaking in the presence of primary antibodies, followed by washing and incubation with conjugated secondary antibodies (IRDye® 800CW or IRDye® 680RD) for 2 h at room temperature. The immunoblotting results were analyzed using the LI-COR Odyssey Dual-Color System (LI-COR, USA) and ImageJ v.1.8.0.

Cell fractionation

Nuclear and cytoplasmic separation experiments followed the methods outlined in previous experiments (Chang et al., 2021). Subsequently, the cellular fractions were subjected to

western blotting.

Immunocytochemistry and immunohistochemistry

For immunocytochemistry, cultured cells were fixed with 4% paraformaldehyde for 15 min, followed by permeabilization with 0.5% Triton X-100 for 30 min and blocking with 5% bovine serum albumin for 1 h at room temperature. Subsequently, the cells were incubated with specific primary antibodies at 4°C overnight, then washed and incubated with conjugated secondary antibodies for 2 h at room temperature or overnight at 4°C. Following sealing with a fluorescence quencher, the cells were analyzed using LSM880 META confocal microscopy and software (Carl Zeiss Meditec AG, Germany).

For immunohistochemistry, dorsal skin samples from various treatment groups were excised and fixed overnight in 4% paraformaldehyde. Samples were cryoprotected by sequential dehydration in 15% and 30% sucrose, embedded in OCT compound, and cryosectioned at 8 μ m thickness. The sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome using standard procedures. Dermal thickness, skin roughness, and collagen content were subsequently quantified using ImageJ (v.1.8.0). To assess skin roughness, the epidermis was outlined, and the maximum height differences between adjacent peaks and valleys were measured in each slice.

Quantitative real-time PCR (qPCR)

Total RNA was extracted using AG RNAex Pro Reagent in accordance with the manufacturer's instructions. cDNA was synthesized from 1 000 ng of RNA using a commercial reverse transcription kit. Quantitative PCR amplification was performed in a 30 μ L reaction volume using gene-specific primers and SYBR Green PCR Mix on a real-time PCR system (CFX Connect™ Optics Module, Bio-Rad Laboratories, USA). Gene expression was normalized to GAPDH expression, and fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are provided in Supplementary Table S5.

Detection of cellular ATP and cAMP levels

Cellular ATP levels in skin tissue were measured using an ATP bioluminescence assay kit (Beyotime Institute of Biotechnology, China), following the manufacturer's instructions. Briefly, skin samples were washed with PBS and homogenized in 160 μ L of lysis buffer. Following lysis, the cells were centrifuged at 4°C for 5 min at 13 500 $\times g$, and 50 μ L of supernatant was collected and mixed with 100 μ L of ATP detection solution. Finally, cellular ATP levels were determined using an Infinite 200 Pro microplate reader (TECAN, Switzerland).

For cAMP quantification, skin tissue was rinsed with PBS and homogenized in ice-cold 0.1 mol/L HCl at a 1:5 ratio (w/v). After centrifugation to remove debris, the supernatants were neutralized with 1 mol/L NaOH and subsequently diluted two-fold with Calibrator Diluent RD5-55. cAMP concentrations were determined using a Mouse cAMP Assay Kit (KGE012B, R&D Systems, USA) following the manufacturer's protocol. Optical density of each well was measured using an Infinite 200 Pro microplate reader (TECAN, Switzerland) set to 450 nm. A standard curve was constructed by plotting the mean absorbance values for each standard, enabling the precise determination of cAMP concentration for each sample.

Cell viability analysis

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8; MCE, USA). Briefly, MSFs were subjected to 8 J/cm² red light once daily for four consecutive days. At each time point, culture medium was replaced with 10% CCK-8 solution, and plates were incubated for an additional 2 h at 37°C. Subsequently, absorbance at 450 nm was measured using an Infinite 200 Pro microplate reader (TECAN, Switzerland).

Flow cytometry

Flow cytometry was performed to assess protein expression in both skin tissue and cultured cells. Dorsal skin samples from different treatment groups were harvested and enzymatically digested with Dispase II, followed by filtration and centrifugation at 250 ×g for 20 min at 4°C to obtain a single-cell suspension. Cells were fixed in 4% paraformaldehyde for 15 min at room temperature, followed by permeabilization with 500 µL of 0.1% Triton X-100 and incubation with the p-AKT and type I collagen antibodies, in accordance with the manufacturer's instructions. Samples were analyzed using a CytoFLEX flow cytometer (Beckman Coulter, USA) and data were processed using FCS Express (De Novo Software, USA).

Enzyme-linked immunosorbent assay (ELISA)

TGFβ1 protein levels in skin tissue homogenates were measured using a commercial mouse TGFβ1 ELISA kit (bll20217, BAILILAI BIOLOGY, China) according to the manufacturer's instructions. Protein concentrations were normalized and reported as pg/mg protein. Absorbance was measured at 450 nm using an Infinite 200 Pro microplate reader (TECAN, Switzerland).

Quantification and statistical analysis

All statistical analyses were conducted using GraphPad Prism v.8.4.2. Paired *t*-tests were utilized for comparisons involving two groups, and one-way analysis of variance (ANOVA) was employed for experiments comprising more than two groups. A threshold of *P* < 0.05 was adopted to ascertain statistical significance for all analyses.

RESULTS

Red light stimulates collagen accumulation in aged skin

To investigate the rejuvenating effects of light exposure, dorsal skin of 18–20-month-old mice was irradiated daily for 28 days with green light (520–525 nm), red light (630–635 nm), or white light (400–800 nm) at an energy density of approximately 8 J/cm² (6.67 mW/cm² for 20 min). Histological analysis revealed that red light, but not white or green light, significantly improved surface roughness and increased dermal thickness in the skin of aged mice (Figure 1A), suggesting a wavelength-specific regenerative effect.

Noninvasive assessments further confirmed red light-induced improvements in skin morphology and physiology. Red light exposure markedly improved the overall skin condition of mice, including enhanced smoothness (Figure 1B), increased elasticity (Figure 1C), and reduced TEWL (Figure 1D), indicating restoration of skin barrier function and mechanical resilience. To investigate the molecular basis of these effects, transcriptome sequencing was performed on skin from red light-treated mice. Differential expression analysis revealed distinct transcriptional signatures between treated and untreated skin, as shown by volcano plot

and heatmap (Supplementary Figure S1A, B). Gene Ontology (GO) enrichment analysis of signature genes showed an up-regulation in processes related to tissue and system development, cell proliferation, cell differentiation, and response to stimuli (Figure 1E). These genes were also enriched in molecular functions such as molecular binding, transmembrane transport, and transcriptional translation (Supplementary Figure S1C). Given the role of collagen organization in skin development and aging (Li et al., 2015), the expression profiles of collagen genes were examined using FPKM quantification. Notably, type I collagen exhibited the most significant changes following red light treatment (Figure 1F). KEGG pathway analysis further showed enrichment of pathways involved in signal transduction, as well as signal molecules and their interactions (Supplementary Figure S1D). These transcriptomic findings were validated by biochemical analyses. Masson's trichrome staining confirmed a significant increase in dermal collagen content in red light-treated mice compared to the untreated controls (Figure 2A). Dermal thickness and skin surface roughness were also significantly improved (Figure 2B; Supplementary Figure S2A). Immunofluorescence and western blot analysis showed increased type I collagen protein levels in treated skin compared to untreated skin (Figure 2C; Supplementary Figure S2B–D). At the transcript level, red light treatment enhanced the mRNA levels of type I collagen and connective-tissue growth factor (CTGF), a key upstream regulator of type I collagen production, in aged skin, which were significantly lower than levels in young dermis (Figure 2D, E). These results suggest that red light counteracts age-associated collagen loss, contributing to the visible improvement in skin appearance and integrity.

Dermal fibroblasts are the principal source of type I collagen in mouse skin (Li et al., 2015) and play a central role in cutaneous remodeling (Dees et al., 2020). To explore the specific molecular mechanism by which red light promotes collagen generation, type I collagen expression was assessed in skin fibroblasts from 6–8-month-old mice (MSFs) and mouse embryos (MEFs). Results showed that red light exposure induced a dose-dependent increase in type I collagen mRNA levels in MSFs, with significant up-regulation observed at doses of 2, 4, and 8 J/cm² (Figure 3A). The 8 J/cm² dose was selected for subsequent experiments based on its maximal effect and lack of thermal injury (Supplementary Figure S3). Western blot analysis demonstrated increased type I collagen protein levels in MSFs following 12 h of red light exposure, with peak expression observed after 24 h (Figure 3B). Immunofluorescence staining confirmed red light-induced promotion of type I collagen expression in both MSFs (Figure 3C; Supplementary Figure S4A) and MEFs (Figure 3D; Supplementary Figure S4B). Fibroblast proliferation was also significantly enhanced in MSFs (Figure 3E), MEFs (Supplementary Figure S4C), and skin tissue (Figure 3F; Supplementary Figure S4D) following red light exposure, indicating increased fibroblast activity both *in vitro* and *in vivo*. These findings suggest that red light stimulates fibroblast proliferation and promotes type I collagen synthesis, contributing to its age-defying effects in aged skin.

Red light-induced type I collagen accumulation requires activation of TGFβ and AKT in dermal fibroblasts

To identify the molecular mediators responsible for red light-induced collagen synthesis, two key signaling pathways were

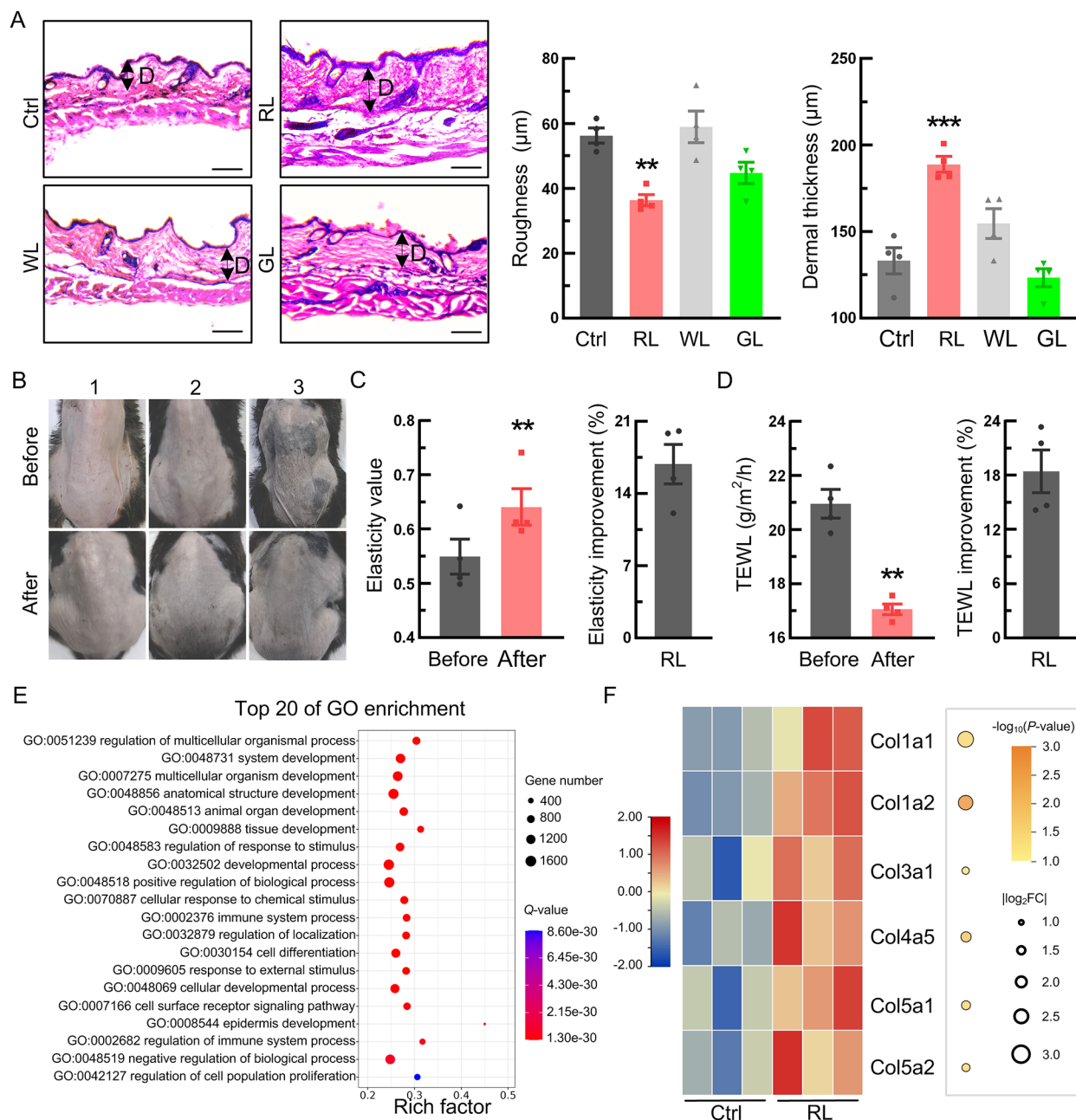


Figure 1 Red light stimulates epigenetic and genomic changes in aging skin

A: Hematoxylin-eosin staining of skin sections from 18–20-month-old mice was performed following 28 days of treatment with green (520–525 nm, GL), red (630–635 nm, RL), or white light (400–800 nm, WL). Skin roughness and dermal thickness were quantified using ImageJ. Double-headed arrows indicate dermal thickness. D, dermis; scale bar: 100 μm ; $n=4$. **B:** Photographic assessment of dorsal skin of mice before and after 28 days of red light exposure. **C, D:** Histograms illustrating dorsal skin parameters of elasticity (**C**) and transepidermal water loss (TEWL) (**D**) at day 0 and after 28 days of red light exposure. $n=4$. **E:** Enriched GO terms for biological processes following red light treatment in 18–20-month-old mice skin. $n=3$ mice in each group. **F:** Heatmap showing collagen expression levels of differentially expressed genes (DEGs) in the skin of 18–20-month-old mice. $n=3$ mice in each group. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$, paired t -test for two groups. Data are mean $\pm$ SEM.

examined. TGF β -dependent SMAD3 phosphorylation, which plays a critical role in collagen gene expression (Sacchetti et al., 2017; Zehender et al., 2021), and AKT, a key molecule involved in photoregulated proliferation (Huang et al., 2013; Liang et al., 2012; Zhang et al., 2009).

Skin tissue showed a significant 50%–60% decrease in the mRNA levels of TGF β 1, a major isoform regulating collagen (Györfi et al., 2018; Meng et al., 2016; Moreau et al., 2022; Quan et al., 2010; Wan et al., 2023), in aged individuals compared to the younger cohort, while red light treatment

partially restored these levels by 20%–30% (Figure 4A). ELISA analysis confirmed the increase in TGF β 1 protein levels in red light-treated aged skin (Figure 4B), as well as in CD45 $^{+}$ CD11b $^{+}$ F4/80 $^{+}$ macrophages (Supplementary Figure S5A), cytokeratin 5-positive skin keratinocytes (Supplementary Figure S5B), and type I collagen-positive skin fibroblasts (Supplementary Figure S5C). Fibroblast-derived autocrine TGF β acts as a rate-limiting factor in collagen synthesis by activating TGF β receptor signaling through TGF β R1 and TGF β R2 (Dees et al., 2020; Györfi et al., 2018).

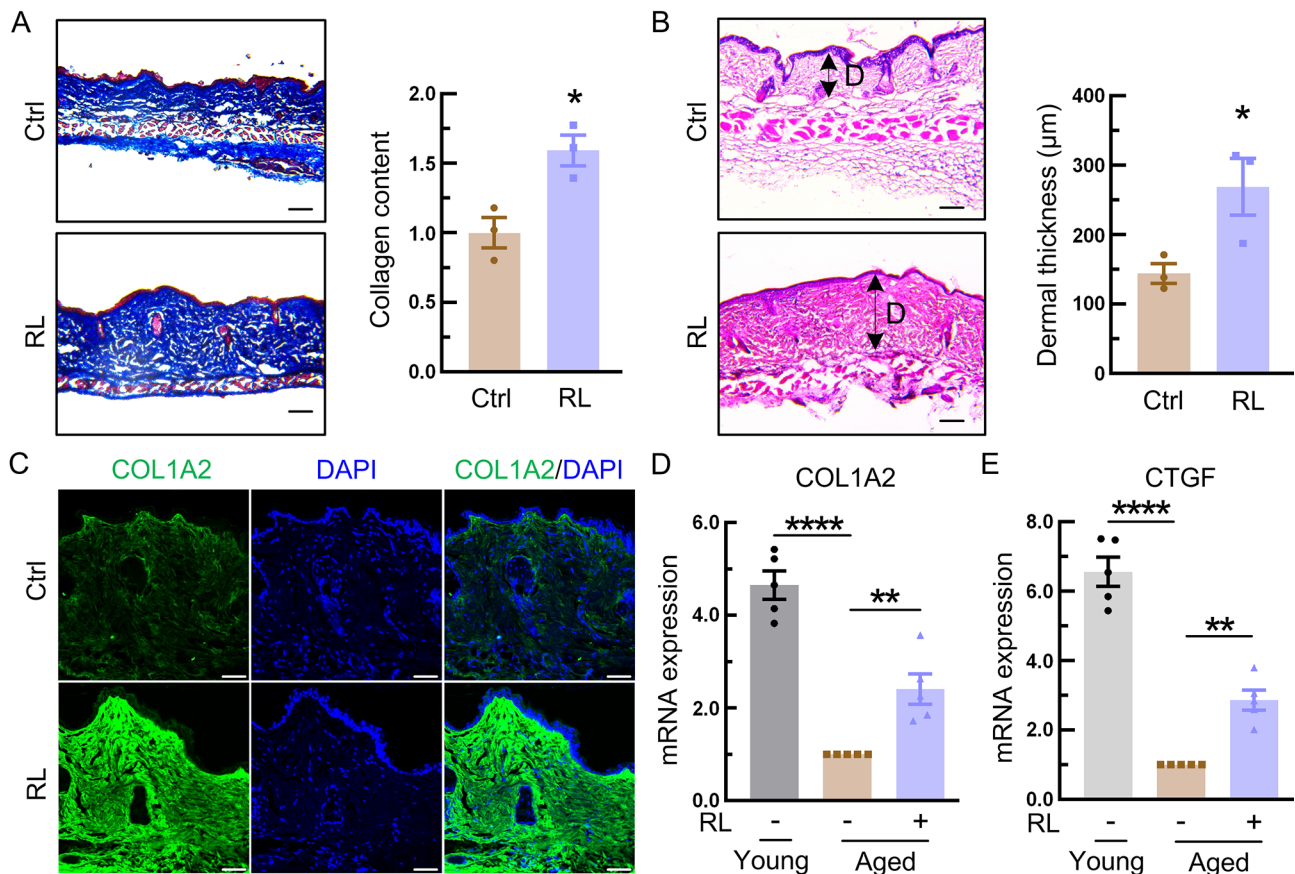


Figure 2 Red light promotes type I collagen expression in aged mice

A: Masson's trichrome staining of skin sections from aged mice following 28 days of red light treatment. Dermal collagen is stained blue, nuclei are stained black, and muscle fibers are stained red. Quantification of collagen fibril staining (blue) per microscopic field was performed using ImageJ. Scale bar: 100 μ m. $n=3$. B: Hematoxylin-eosin staining of skin sections from aged mice treated with red light for 28 days. Quantification of skin roughness and dermal thickness was performed using ImageJ. Double-headed arrows indicate dermal thickness. D, dermis; scale bar: 100 μ m. $n=3$. C: Immunofluorescence was employed to detect type I collagen expression in the skin tissue of aged mice following 28 days of red light treatment. Scale bar: 50 μ m. D, E: mRNA levels of type I collagen (D) and CTGF (E) in skin tissue from aged mice following 28 days of red light treatment compared to those in the skin of young mice. *: $P<0.05$; **: $P<0.01$; ****: $P<0.0001$, one-way ANOVA for multiple groups or paired t -test for two groups. Data are mean $\pm$ SEM.

This process involves the formation of a heterotetrameric receptor complex upon TGF β binding (Moreau et al., 2022), which initiates a downstream signaling cascade. To assess whether red light influences this pathway, TGF β R1 activity was further investigated. Western blot analysis revealed a significant increase in the phosphorylation of TGF β R1 in the skin of aged mice following red light exposure (Figure 4C). Pharmacological inhibition of TGF β R1 using SB435142, along with AAV-mediated knockdown via AAV-shTGF β R1, suppressed red light-induced up-regulation of type I collagen, as evidenced by western blotting (Figure 4C) and immunofluorescence (Figure 4D; Supplementary Figures S6, S7A, B). Furthermore, inhibition of TGF β R1 reduced the increases in dermal thickness and skin smoothness induced by red light treatment (Figure 4E; Supplementary Figure S8A–C). Likewise, the impact of AKT on regulating red light-induced type I collagen expression was also examined. Red light exposure led to increased phosphorylation of AKT in aged skin (Supplementary Figure S9A). However, administration of either AAV-shAKT or the AKT inhibitor API-2 significantly reduced type I collagen expression following red light treatment (Figure 4D; Supplementary Figures S7A, S9B). Inhibition of AKT similarly abolished red light-induced

enhancements in dermal thickness and skin smoothness (Figure 4E; Supplementary Figures S8A, B, S9C). In addition, AKT inhibition markedly impeded the proliferation of COL1A2⁺ fibroblasts induced by red light exposure (Figure 4F; Supplementary Figure S9D). These findings highlight the essential role of TGF β and AKT in mediating red light-induced expression of type I collagen and refinement of skin surface aesthetics.

Red light promotes type I collagen transcription by activating the TGF β /SMAD signaling pathway

The canonical TGF β -SMAD signaling pathway is a well-established regulator of collagen synthesis (Quan et al., 2021; Wan et al., 2023). To determine whether this signaling cascade mediates red light-induced collagen expression, the activation and nuclear translocation of SMAD proteins were assessed. After activation, SMAD2/3 combines with SMAD4 to form a complex, which then translocates into the nucleus to regulate downstream target gene expression (Moreau et al., 2022; Sacchetti et al., 2017). Accordingly, immunofluorescence analysis revealed that red light increased the phosphorylation of SMAD2/3 and SMAD4, facilitating their interaction and subsequent nuclear translocation in MSFs (Figure 5A) and MEFs (Supplementary Figure S10A). These

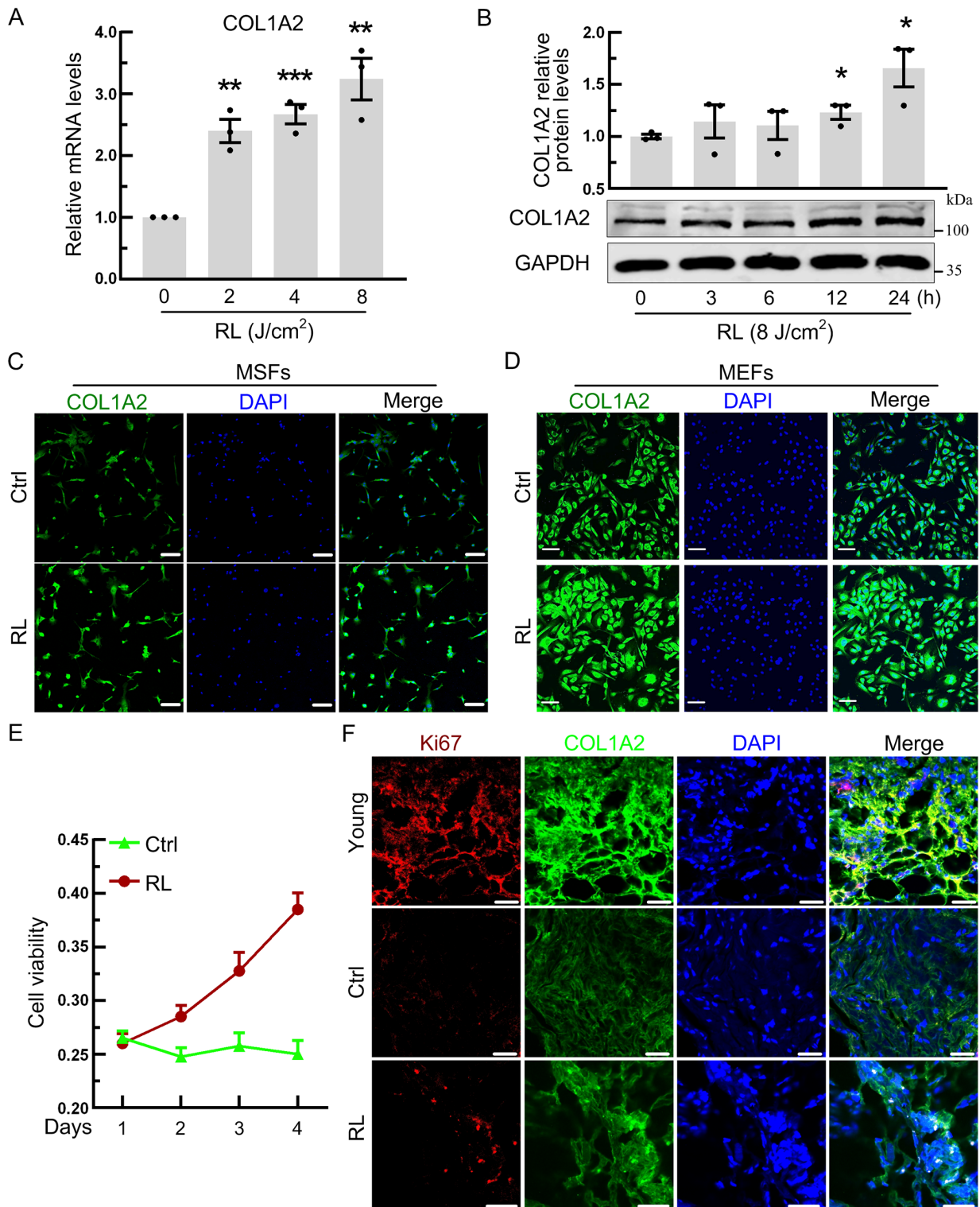


Figure 3 Red light promotes type I collagen expression in MSFs and MEFs

A: qPCR analysis of COL1A2 expression in MSFs following treatment with different doses of red light. B: Western blot analysis of type I collagen protein levels in MSFs following 8 J/cm² red light treatment at different time points. C, D: Immunofluorescence analysis was conducted to assess type I collagen levels in MSFs (C) and MEFs (D) after 8 J/cm² red light treatment for 24 h. Scale bar: 100 µm. E: Proliferation capacity of MSFs was assessed using the CCK-8 assay, with daily red light treatment administered over four consecutive days. F: Ki67 staining was used to assess COL1A2⁺ cell proliferation in aged mouse skin following 28 days of 8 J/cm² red light treatment. Scale bar: 30 µm. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$, one-way ANOVA for multiple groups or paired t -test for two groups. Data are mean ± SEM.

effects were attenuated by pre-treatment with the TGFβR1 inhibitor SB431542. Consistently, in aged skin tissue, red light enhanced the phosphorylation of SMAD2/3 and subsequent expression of type I collagen, with both responses effectively

suppressed by SB431542 (Figure 5B, C; Supplementary Figure S10B, C). These findings confirm that red light activates the TGFβ/SMAD signaling axis to promote type I collagen transcription, supporting its role as a key regulatory

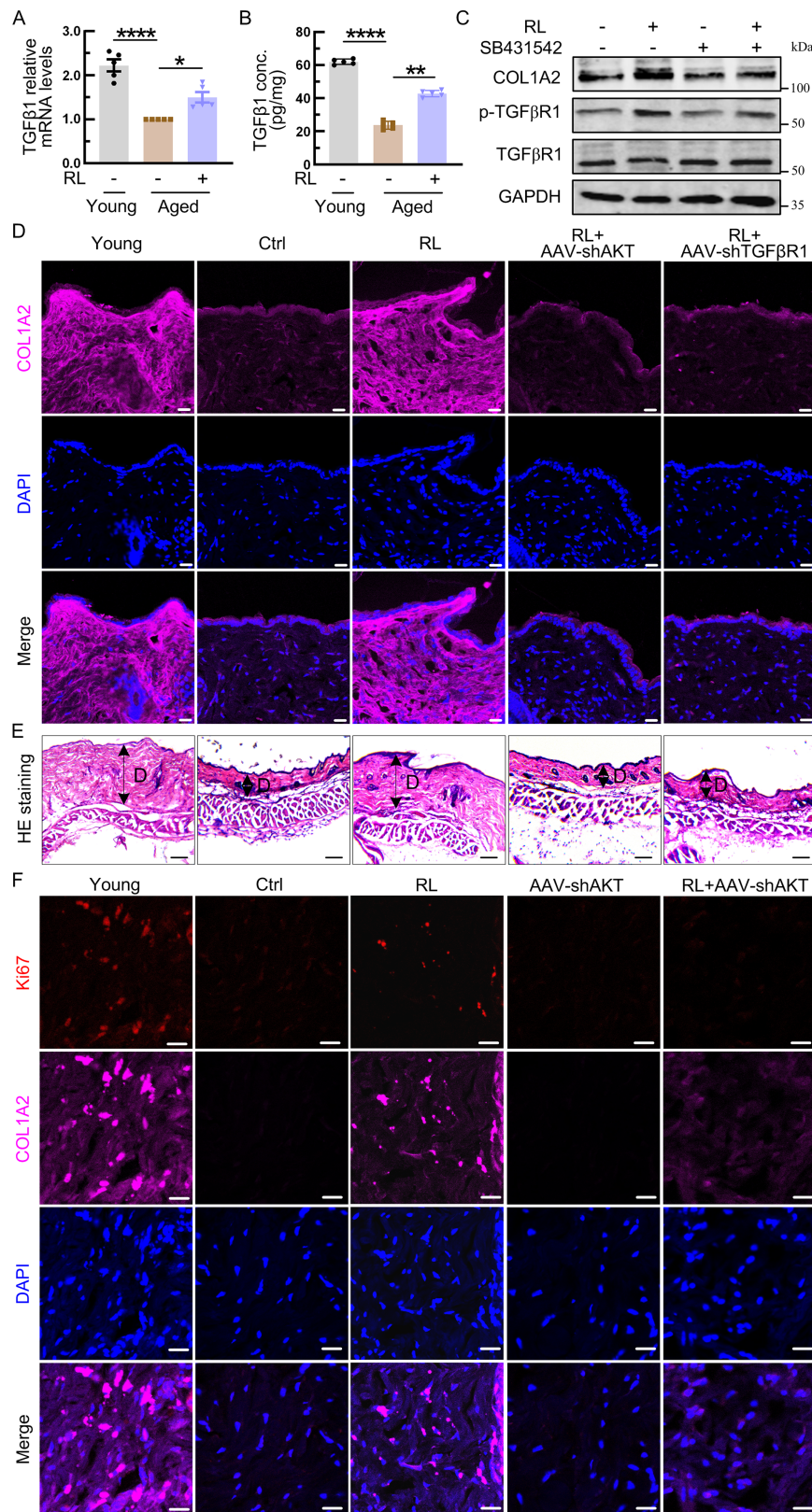


Figure 4 Red light promotes type I collagen expression via TGFβ and AKT activation

A: qPCR analysis of TGFβ1 mRNA expression in mouse skin tissues following red light treatment. $n=5$. B: ELISA of TGFβ1 secretion in mouse skin tissues following red light treatment. $n=5$. C: Representative western blot analysis showing p-TGFβR1 and type I collagen levels in aged mice treated with red light, SB431542, or both. D: Representative immunofluorescence images showing type I collagen expression in skin sections from aged mice treated with red light, AAV-shAKT/AAV-shTGFβR1, or both. Scale bar: 50 μm. E: Hematoxylin-eosin staining of skin sections obtained from aged mice treated with red light, AAV-shAKT/AAV-shTGFβR1, or both. Double-headed arrows indicate dermal thickness. D, dermis; scale bar: 100 μm. F: Proliferation of COL1A2⁺ cells was assessed using Ki67 staining in aged mouse skin treated with red light, AAV-shAKT, or both. Scale bar: 30 μm. *: $P<0.05$; **: $P<0.01$; ****: $P<0.0001$, one-way ANOVA for multiple groups or paired t -test for two groups. Data are mean±SEM.

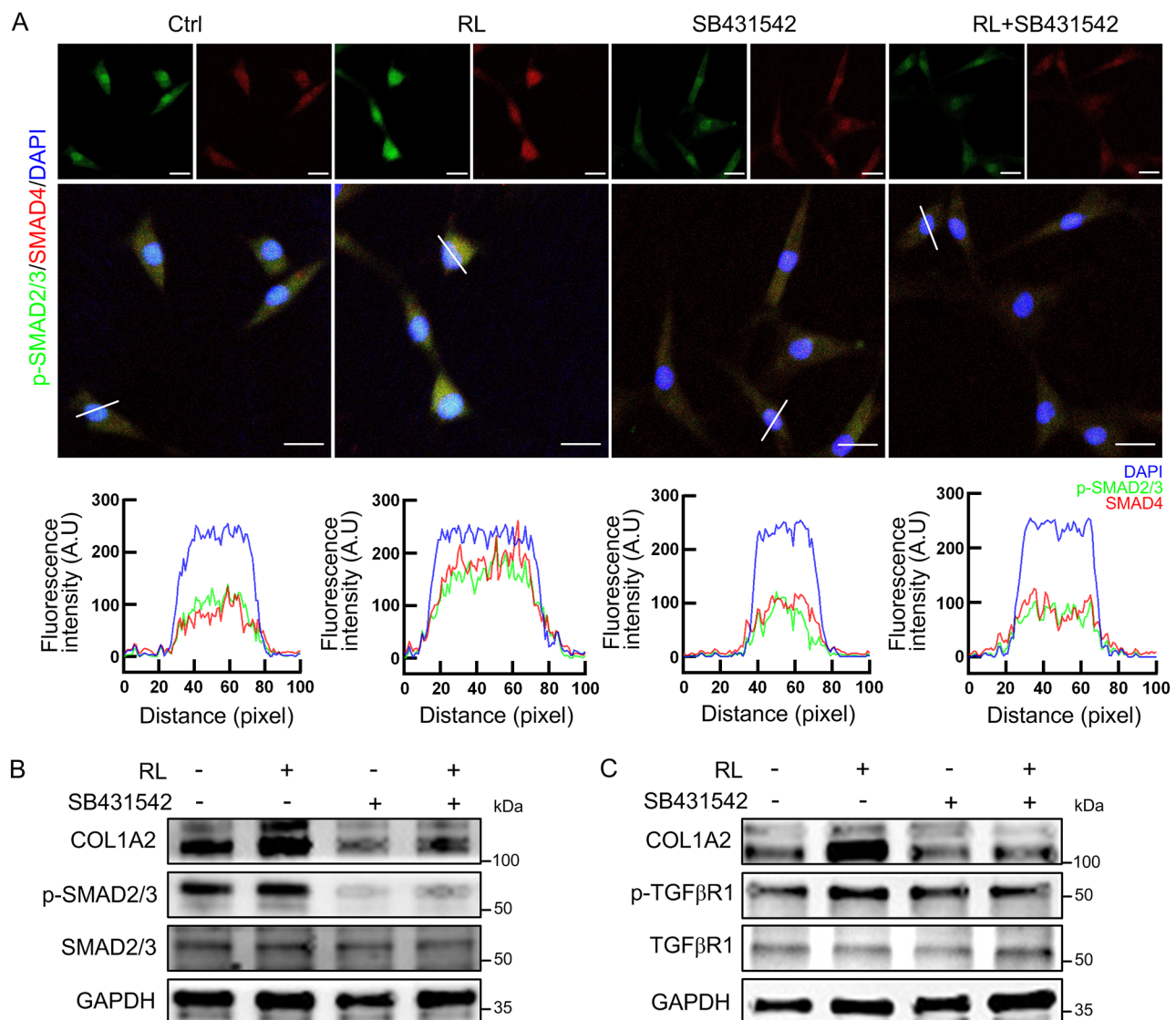


Figure 5 Red light promotes type I collagen expression via TGFβ/SMAD signaling pathway activation

A: Fluorescence colocalization analysis of nuclear translocation of SMAD2/3 and SMAD4 in MSFs following red light treatment, with or without SB431542 (60 μmol/L). A line was drawn for selected cells, and fluorescence intensity of different channels along the line was quantitatively analyzed using LSM880 META. Scale bar: 20 μm. B, C: Representative western blot assays of type I collagen, p-SMAD2/3 (B), and p-TGFβR1 (C) expression in skin tissue from aged mice treated with red light, SB431542, or both.

pathway underlying red light-induced dermal remodeling in aging skin.

Red light inhibits type I collagen degradation by activating the AKT/NRF2/MMP signaling pathway

The mechanism by which red light-activated AKT induces type I collagen expression was further explored. In aging fibroblasts, increased ROS levels during the late proliferative stages have been linked to impaired cellular function (Lee et al., 2002), while proliferative activity has been shown to mitigate oxidative stress (Mei et al., 2022). The NRF2/HO-1 axis orchestrates cellular defense by alleviating oxidative damage; however, its activity is diminished in aged mice (Lee et al., 2021a). In agreement with these reports, reduced NRF2 and HO-1 mRNA levels were observed in aged mouse skin compared to young controls (Figure 6A, B). However, red light exposure significantly reversed this age-associated decline. To investigate the association between AKT activation and NRF2/HO-1 signaling, AKT was inhibited using API-2. Western blot analysis demonstrated that AKT inhibition

significantly reduced the expression of NRF2 and HO-1 in aged mice (Figure 6C; Supplementary Figure S11). Newly synthesized NRF2 translocates into the nucleus, where it binds to antioxidant response elements (AREs) to activate transcription of target genes, including HO-1 (Hiebert et al., 2018; Loboda et al., 2016). Immunofluorescence imaging showed that red light promoted nuclear translocation of NRF2 in both MSFs and MEFs, an effect attenuated by treatment with the AKT inhibitor API-2 (Supplementary Figure S12A, B). These results were further validated in aged skin tissue using nuclear-cytoplasmic fractionation (Figure 6D; Supplementary Figure S12C). The administration of HO-1i, a specific HO-1 inhibitor, significantly suppressed the red light-induced expression of type I collagen in aged mice (Figure 6E, F; Supplementary Figure S12D, E). Together, these findings indicate that the AKT-dependent activation of the NRF2/HO-1 axis is essential for promoting type I collagen expression in response to red light in aged skin.

The NRF2/HO-1 axis is recognized for its downregulation of

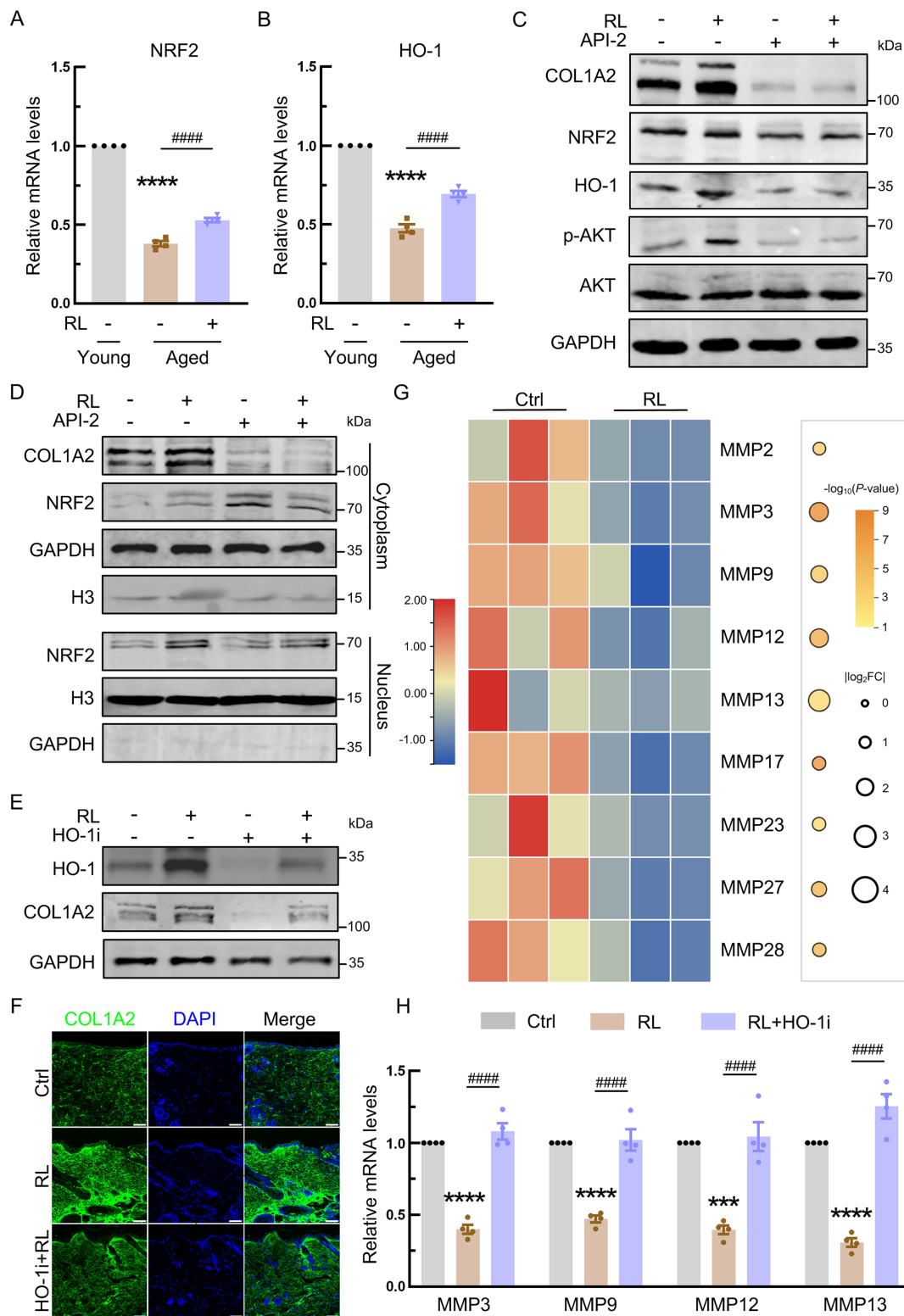


Figure 6 Red light inhibits type I collagen degradation via AKT/NRF2/HO-1 signaling pathway activation

A, B: qPCR analysis of NRF2 (A) and HO-1 (B) mRNA expression in mouse skin tissue following red light treatment. $n=4$. C: Representative western blot assays of p-AKT, HO-1, NRF2, and type I collagen expression in aged mice treated with red light, API-2, or both. D: Western blot analysis of nuclear-cytoplasmic separation in skin tissue. Cytosolic and nuclear extracts were immunoblotted for NRF2, while GAPDH and histone H3 proteins were probed to confirm thorough separation of the cytosolic and nuclear fractions. E: Representative western blot analysis of type I collagen expression in skin tissue after red light treatment with or without HO-1i. F: Representative immunofluorescence images of type I collagen in skin sections from aged mice after 28 days of red light treatment with or without HO-1i. Scale bar: 50 μm . G: Heatmaps showing relative expression levels of DEGs involved in various MMPs. $n=3$. H: qPCR analysis of MMP3, MMP9, MMP12, and MMP13 mRNA expression in mouse skin tissue following red light treatment, with and without HO-1i. ****: $P<0.001$; *****: $P<0.0001$ compared to control group, and ####: $P<0.0001$ compared to indicated groups, one-way ANOVA for multiple groups. Data are mean $\pm$ SEM.

matrix metalloproteinases (MMPs) (Tertil et al., 2015; Wu et al., 2016)—key enzymes responsible for collagen degradation—supporting its involvement in maintaining collagen homeostasis. In aged skin, red light treatment led to a significant decrease in the expression of multiple MMPs relative to untreated controls (Figure 6G). Notably, the suppression of MMP expression by red light, defined by a $|\log_2\text{FC}|$ exceeding 2, was significantly reversed upon HO-1i administration (Figure 6H). These findings substantiate the distinct role of the AKT/NRF2/MMP signaling pathway in mediating red light-dependent preservation of type I collagen in aging skin.

Red light-induced AKT activation requires cAMP generation

Red light regulates multiple cellular processes by stimulating intracellular signaling pathways through the activation of photoreceptor molecules, such as cytochrome c enzyme, leading to the generation of second messenger molecules, including Ca^{2+} , ROS, and cAMP (Chang et al., 2021; Gu et al., 2017; Zhang et al., 2020). Among these, only cAMP was found to significantly mediate red light-induced AKT activation in aged mice, as demonstrated using the adenylyl cyclase inhibitor, SQ22536 (Supplementary Figure S13A), which suppressed cAMP generation from ATP in MSFs (Supplementary Figure S13B). Indeed, red light exposure substantially increased both ATP and cAMP levels in aged

skin tissue (Figure 7A, B). Western blot analysis further showed that red light-stimulated AKT phosphorylation was inhibited by SQ22536 (Figure 7C; Supplementary Figure S13C), accompanied by a concurrent reduction in type I collagen expression. Immunofluorescence further substantiated that inhibiting cAMP production diminished red light-induced type I collagen expression in aged mice (Supplementary Figure S14A, B). To assess downstream effects, mRNA levels of NRF2, HO-1, MMP3, MMP9, MMP12, and MMP13 were analyzed. Red light significantly up-regulated their expression, but these effects were largely inhibited by SQ22536 (Figure 7D). Importantly, red light-induced improvements in dermal thickness and smoothness were also reversed by SQ22536 in aged mice (Figure 7E; Supplementary Figure S14C, D). Collectively, these findings strongly indicate that red light-generated cAMP production is essential for AKT activation and its downstream signaling cascade, including the up-regulation of type I collagen and structural improvement of aging skin.

Red light-induced activation of dermal TGF β and AKT signaling initiates epidermal renewal

The basement membrane at the dermal-epidermal junction is structurally supported by the interweaving of dermal collagen fibrils with epidermal anchoring fibers, maintaining tissue cohesion and barrier integrity (Rousselle et al., 2019). Collagen fibrils not only reinforce structural adhesion but also

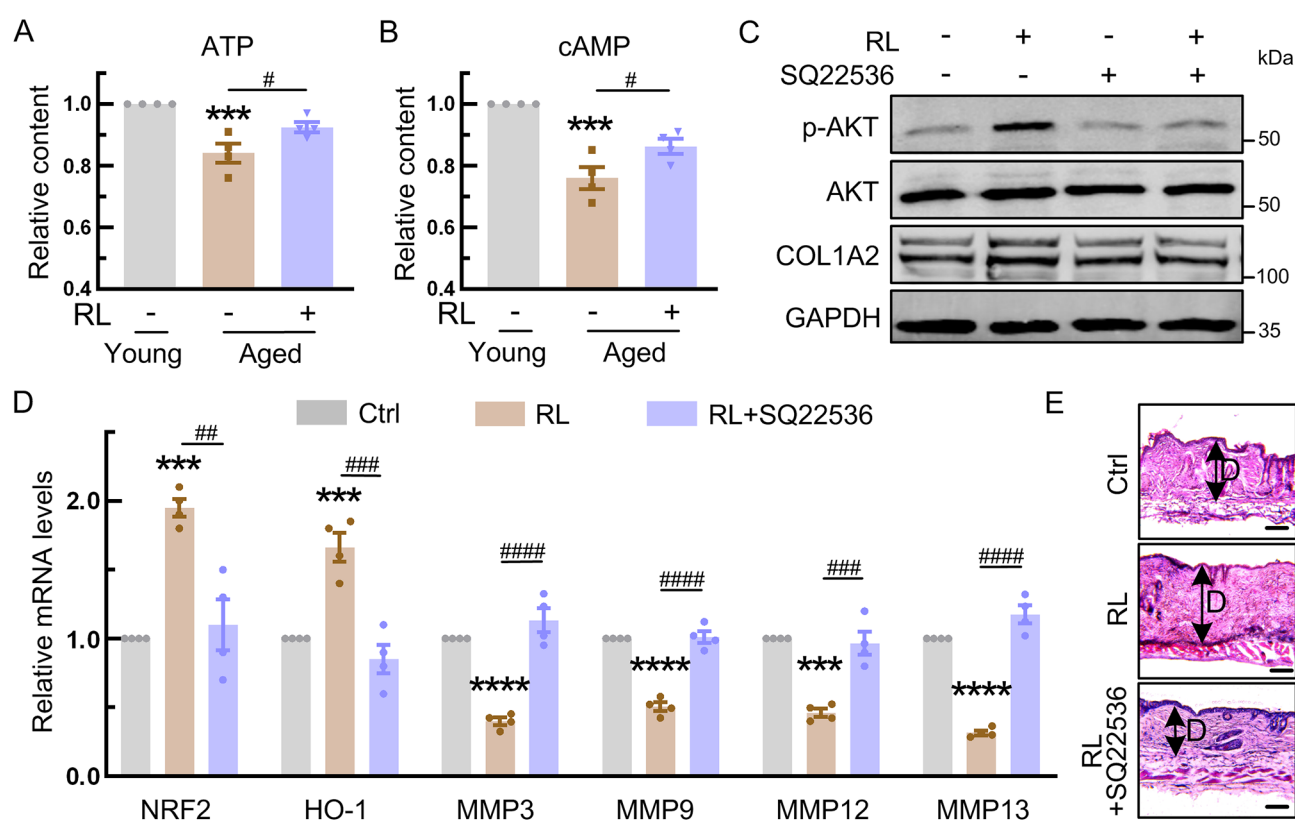


Figure 7 Red light-induced AKT activation is dependent on cAMP generation

A, B: Relative ATP (A) and cAMP (B) levels were quantified in skin tissue following red light treatment. $n=4$. C: Representative western blot analysis of p-AKT and type I collagen expression in skin tissue subjected to different treatments. D: qPCR analysis of NRF2, HO-1, MMP3, MMP9, MMP12, and MMP13 mRNA expression in mouse skin tissue following red light treatment, with and without SQ22536. $n=4$. E: Hematoxylin-eosin staining of skin sections obtained from aged mice treated with red light, SQ22536, or both. Double-headed arrows indicate dermal thickness. D, dermis; scale bar: 100 μm . ***: $P<0.001$; ****: $P<0.0001$ compared to control group, and #: $P<0.05$; #: $P<0.01$; ###: $P<0.001$; ####: $P<0.0001$ compared to indicated groups, one-way ANOVA for multiple groups. Data are mean $\pm$ SEM.

promote epidermal renewal by stimulating keratinocyte proliferation and differentiation—processes essential for sustaining epithelial homeostasis (Gretzmeier et al., 2022). This signaling is transduced, in part, by integrin $\alpha 2 \beta 1$, a collagen I receptor highly expressed on basal keratinocytes, which mediates responses to the surrounding extracellular matrix (Pang et al., 2023; Schulz et al., 2015). To investigate whether red light modulates this axis, the expression of phosphorylated integrin $\beta 1$ (p-integrin $\beta 1$) in the epidermis was evaluated. Immunofluorescence staining revealed a marked up-regulation of p-integrin $\beta 1$ in response to red light exposure (Figure 8A; Supplementary Figure S15A). This response was effectively abolished by the suppression of TGF β R1 or AKT, suggesting both signaling pathways are responsible for the red light-induced activation of integrin. Notably, p-integrin $\beta 1$ levels were significantly reduced in aged mice compared to young controls. Further analysis demonstrated that red light enhanced keratinocyte proliferation and differentiation, as indicated by increased Ki67 and cytokeratin 10 expression, respectively (Figure 8B, C; Supplementary Figure S15B, C). Importantly, these effects were counteracted by AAV-mediated knockdown or pharmacological inhibition of TGF β R1 and AKT, confirming the dependence of epidermal renewal on these signaling pathways. These findings suggest that red light stimulates keratinocyte proliferation and differentiation via activation of the TGF β and AKT signaling pathways.

DISCUSSION

Recent clinical studies have established the therapeutic promise of red light in ameliorating age-related skin degeneration, with documented improvements across structural, mechanical, and aesthetic parameters. For instance, Couturaud et al. (2023) reported marked enhancements in dermal elasticity, density, and surface smoothness, accompanied by reductions in periorbital wrinkles, in 20 participants following red light therapy. Similarly, Cannarozzo et al. (2021) reported significant wrinkle reduction in 22 participants, evaluated using the Modified Fitzpatrick Wrinkle Scale, with only mild erythematous rash observed in two cases. Notably, combinatorial interventions, such as red light with near-infrared light (630 nm/850 nm), showed improved hydration, elasticity, and density in 30 participants without affecting thyroid function, confirming its safety and efficacy (Lee et al., 2021b). Additional integration with fractional CO₂ laser has also been shown to synergistically enhance wrinkle resolution and patient satisfaction (Nistico et al., 2021). Collectively, these findings highlight the potential of red light therapy as a non-invasive modality for skin rejuvenation, while also emphasizing the need for further research on its underlying mechanisms and efficacy with other wavelengths.

Expanding upon these clinical findings, this study systematically explored the molecular and cellular pathways through which red light exerts its regenerative effects on aging skin. Compared to other wavelengths, red light exhibited superior efficacy in promoting the production and stability of type I collagen. Mechanistic analyses in both cellular and animal models demonstrated that red light activates TGF β /SMAD signaling to drive collagen gene transcription, while concurrently engaging AKT/NRF2/MMP signaling-mediated collagen protein stability. These dual regulatory mechanisms not only restored dermal

architecture—evidenced by increased collagen deposition and dermal thickening—but also enhanced epidermal self-renewal and skin smoothness. These findings provide a robust foundation for understanding how red light alleviates age-related collagen loss and support its clinical efficacy in combating skin degeneration.

The sustained up-regulation of type I collagen in dermal fibroblasts following red light exposure reflects multifaceted regulatory mechanism encompassing transcriptional activation, protein stabilization, and enhanced fibroblast proliferation. This distinguishes red light from conventional anti-aging agents, such as proline and ascorbic acid, which primarily act at the transcriptional level (Boo, 2022; Karna et al., 2020), and coenzyme Q10, which targets protein stability (Marcheggiani et al., 2021). Notably, red light retains efficacy in promoting collagen synthesis even under conditions of diminished TGF β R2 expression (Fisher et al., 2016), owing to its activation of the AKT/NRF2/HO-1 pathway, which serves as an alternative regulatory mechanism. In our model, sustained red light exposure at 8 J/cm² over 28 days yielded marked improvements in dermal structure and skin appearance, underscoring its therapeutic potential. TGF β and AKT emerged as reliable molecular indicators of red light efficacy, offering a framework for monitoring and optimizing phototherapeutic protocols. The well-characterized molecular targets and defined exposure parameters contribute to the safety and effectiveness of red light-based interventions in clinical settings.

Clinically, photobiomodulation using light within the 400–1 000 nm spectrum is widely employed to alleviate inflammation, relieve pain, and promote wound healing (Chang et al., 2021). Cytochrome C oxidase, a key component of the mitochondrial respiratory chain, is the principal intracellular photoreceptor for red light (Sinha, 2020). Upon activation, it triggers the production of various signaling molecules, including ROS and cAMP. In the context of aging skin, where elevated ROS levels and diminished cAMP concentrations are hallmarks of cellular dysfunction (Kelly, 2018; Mossad et al., 2022), red light appears to restore signaling equilibrium. Notably, the red light-induced elevation of cAMP may compensate for age-related declines in intracellular signaling fidelity. Functionally, cAMP engages downstream effectors, such as cAMP-dependent protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac), thereby activating the PI3K/AKT signaling pathway (E et al., 2019; Kayhan et al., 2019; Zhao et al., 2019). In the current study, red light markedly activated AKT in aged dermal fibroblasts, which up-regulated the NRF2/HO-1 pathway, an endogenous anti-oxidant system that mitigates oxidative stress and suppresses MMP activation (Kim et al., 2019; Moldogazieva et al., 2018), consequently increasing type I collagen expression. These findings suggest a scenario in which cAMP functions as an initiating signaling molecule that sustains type I collagen-driven tissue remodeling by activating AKT.

TGF β mRNA expression is modulated by diverse pathological conditions in the skin, including sclerosis, wound repair, and aging, through multiple upstream pathways, such as STAT3, FGF2/Snai2, and SOD3 (Bhandari et al., 2020; Koike et al., 2020; Lee et al., 2021a). In aging dermal fibroblasts, oxidative stress has been shown to disrupt TGF β /SMAD signaling (He et al., 2014). Here, red light restored TGF β mRNA expression in aged skin, suggesting

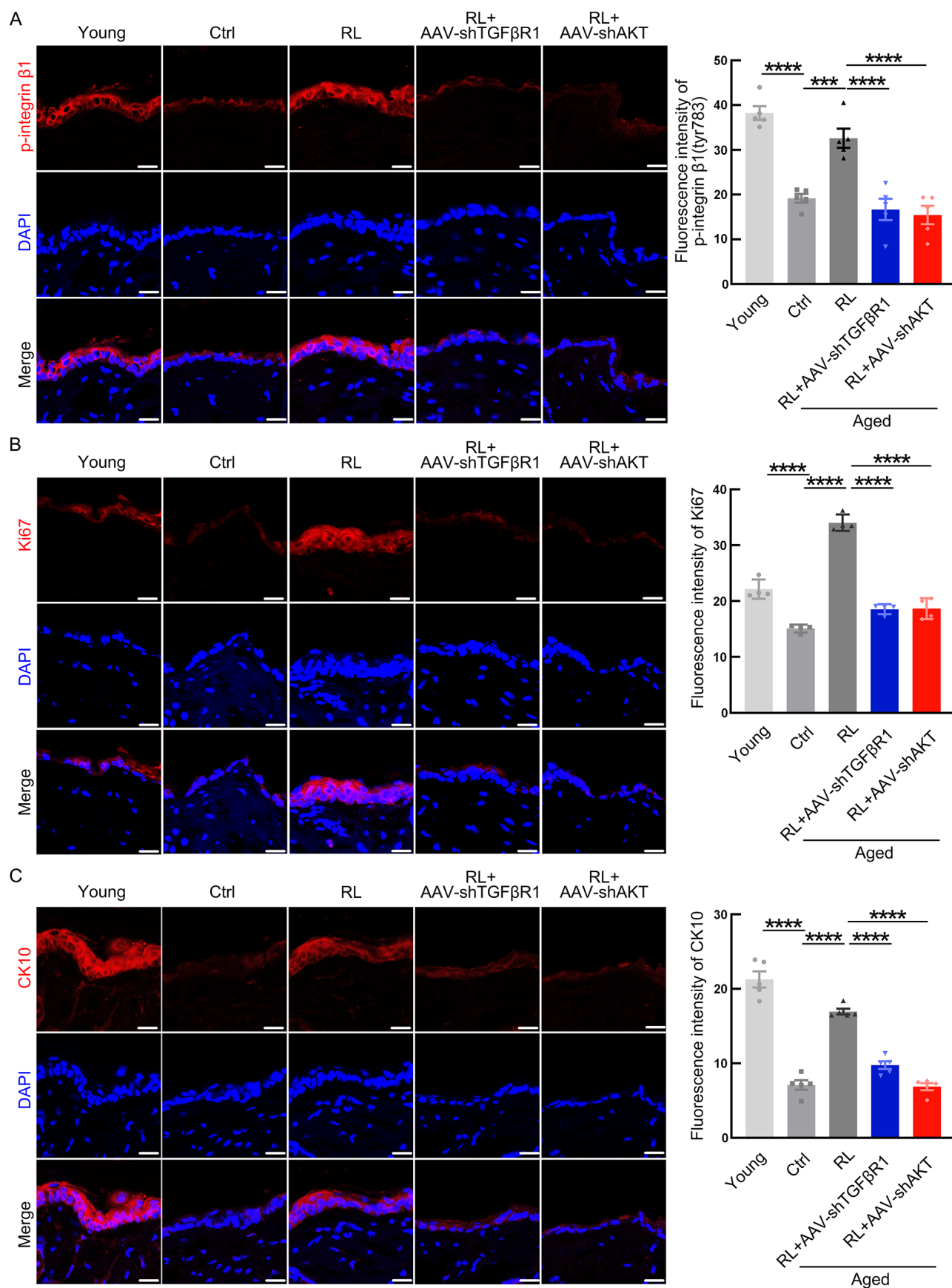


Figure 8 Red light initiates epidermal renewal by activating dermal TGFβ and AKT signaling

A–C: Expression of p-integrin β1 (A), Ki67 (B), and CK10 (C) was evaluated in skin tissues of aged mice after 28 days of 8 J/cm² red light treatment, with or without concurrent subcutaneous injections of AAV-shAKT and AAV-shTGFβR1. Scale bar: 20 μm; *n*=5 for A and C, *n*=4 for B. ***: *P*<0.001; ****: *P*<0.0001, one-way ANOVA for multiple groups. Data are mean±SEM.

that this effect may be mediated by red light-induced mitigation of oxidative stress. This transcriptional enhancement may underlie the observed increase in collagen content. CTGF, a downstream target of the TGF β /SMAD pathway (Quan et al., 2010, 2021), is known for its up-regulation and role in stimulating type I collagen expression in fibrotic skin diseases (Fisher et al., 2016; Quan et al., 2021). In contrast, our aging skin model exhibited reduced mRNA expression of CTGF, type I collagen, and TGF β , all of which were markedly restored following red light exposure. These results suggest that these signaling mediators are critically involved in the precise transcriptional regulation of type I collagen induced by red light in aged skin.

The maintenance of epidermal homeostasis depends on tightly coordinated control of stem and progenitor cell proliferation, survival, and differentiation (Jin et al., 2023). These processes are governed by signaling cues from both epidermal and dermal compartments (Morgner et al., 2015; McAndrews et al., 2022), with integrin pathways—particularly those involving β 1-containing integrins—serving as key mediators. In our study, red light stimulated epidermal self-renewal by enhancing β 1-containing integrin signaling in epidermal basal cells. This activation was triggered by type I collagen input from the dermis, suggesting that red light not only modifies the structural function of the dermis and epidermis but also bolsters dermis-epidermis cohesion, thereby influencing epidermal cell fate.

This study highlights the therapeutic potential of red light therapy in mitigating skin aging through a mechanistically informed and parameter-optimized approach. Red light demonstrated superior efficacy over blue and white light in reversing age-related skin deterioration. Notably, 28 days of red light exposure at 8 J/cm² led to marked improvements in dermal elasticity and wrinkle reduction. To account for individual variability in treatment responsiveness, molecular profiling of key signaling pathways within skin tissue, using either excised samples or advanced imaging modalities such as fluorescent biosensors (Zhang et al., 2021), can guide the precise customization of irradiation parameters, including dosage and duration. This personalized approach offers a non-invasive and effective alternative to more invasive aesthetic interventions, such as injectable fillers or surgery. Notably, the reliance on endogenous cellular pathways reinforces the safety profile and long-term feasibility of red light therapy across heterogeneous patient populations. Nonetheless, the potential for adverse outcomes, including epithelial hyperplasia, particularly in younger individuals subjected to excessive exposure, underscores the need for rigorous control of therapeutic parameters to minimize risks while optimizing efficacy.

Our experimental approach employed AAV-mediated knockdown and pharmacological inhibition to explore the roles of specific molecular pathways. However, to improve the specificity and durability of target gene inhibition, further investigations utilizing gene knockout model mice are currently under development. *In vivo* analyses demonstrated that red light significantly increased the expression of type I collagen (Figure 2; Figure 3) and TGF β 1 (Figure 4A, B; Supplementary Figure S5). Nevertheless, other collagen isoforms, such as type III (Ozog et al., 2013) and type IV (Bielajew et al., 2020), and additional TGF β isoforms, including TGF β 2 (Kim et al., 2021) and TGF β 3 (Liu et al., 2022), may also contribute to the observed effects. Accordingly, the next phase of investigation

will involve targeted validation of these molecules using gene models to further refine the specificity of our findings.

In summary, red light therapy administered at 8 J/cm² for 28 consecutive days significantly improved dermal thickness and surface smoothness in a physiological aging model, primarily through type I collagen-driven tissue remodeling. Mechanistically, red light promoted type I collagen transcription in fibroblasts via activation of the TGF β /SMAD signaling pathway and concurrently stabilized type I collagen protein through AKT/NRF2/MMP regulation, triggered by cAMP signaling. In parallel, red light promoted fibroblast proliferation, effectively mitigating age-related collagen loss and facilitating collagen-dependent epidermal renewal. A better understanding of the regulatory mechanisms underlying red light may provide promising therapeutic targets for addressing skin degeneration.

DATA AVAILABILITY

The metadata and raw sequencing reads have been deposited in the NCBI database (GSE290637), China National Center for Bioinformation (PRJCA036613), and Science Data Bank databases (DOI: 10.57760/sciencedb.j00139.00176).

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

H.C. conceived the study concept; H.C. and Q.S. designed the study; Y.T., J.T., X.A., and W.O. performed the experiments; Y.T., J.T., X.A., and W.O. analyzed most of the data; H.C. and J.T. analyzed the RNA-seq data; Y.T., W.O., J.T., X.A., and Z.Z. raised the mice; H.C., Q.S., and S.Y. wrote and revised the manuscript. All authors read and approved the final version of the manuscript.

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