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A heterocyclic molecule kartogenin induces collagen synthesis of human dermal fibroblasts by activating the smad4/smad5 pathway

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ABSTRACT

Declined production of collagen by fibroblasts is one of the major causes of aging appearance. However, only few of compounds found in cosmetic products are able to directly increase collagen synthesis. A novel small heterocyclic compound called kartogenin (KGN) was found to stimulate collagen synthesis of mesenchymal stem cells (MSCs). So, we hypothesized and tested that if KGN could be applied to stimulate the collagen synthesis of fibroblasts.

Human dermal fibroblasts in vitro were treated with various concentrations of KGN, with dimethyl sulfoxide (DMSO) serving as the negative control. Real-time reverse-transcription polymerase chain reaction, Western blot, and immunofluorescence analyses were performed to examine the expression of collagen and transforming growth factor beta (TGF-β) signaling pathway. The production of collagen was also tested in vivo by Masson's trichrome stain and immunohistochemistry in the dermis of mice administrated with KGN.

Results showed that without obvious influence on fibroblasts' apoptosis and viability, KGN stimulated type-I collagen synthesis of fibroblasts at the mRNA and protein levels in a time-dependent manner, but KGN did not induce expression of α-skeletal muscle actin (α-sma) or matrix metallopeptidase1 (MMP1), MMP9 in vitro. Smad4/smad5 of the TGF-β signaling pathway was activated by KGN while MAPK signaling pathway remained unchanged. KGN also increased type-I collagen synthesis in the dermis of BALB/C mice.

Our results indicated that KGN promoted the type-I collagen synthesis of dermal fibroblasts in vitro and in the dermis of mice through activation of the smad4/smad5 pathway. This molecule could be used in wound healing, tissue engineering of fibroblasts, or aesthetic and reconstructive procedures.

1. Introduction

Collagen is the major structural component of the dermis. Collagen and extracellular matrix degradation accounts for aging of the skin[1]. Collagen, bovine-derived or human-derived has been recognized as a well-accepted treatment for cosmetic purposes since the 1970s [2]. While collagen [3] and analogous composition filling [4] are the main methods for esthetic surgery, biologic and synthetic collagen-embedded wound dressings are used as skin substitutes [5]. During wound healing phases, the formation of collagen-rich granulation tissue is vital for wound closure [6]. For decades, the logic of filling defects by the corresponding collagen components or fibroblasts transplantation [7,8] is the no-alternative choice for clinical researches.

However, in recent years, an unprecedented approach is to regulate the phenotype of endogenous cells by direct in vivo modulation using drug-like small molecules [9–12]. The use of unbiased phenotypic or pathway-based high-throughput cellular screens of chemical libraries has identified molecules that affect adult stem cell-related processes, such as inhibiting the signaling pathway of tumor and adult stem cells manipulation [13,14]. As the major cellular components in the dermis, could fibroblasts produce more collagen by the application of exogenic small molecules? No related molecules have been reported in literature.

In 2012, a heterocyclic compound called kartogenin (KGN) was discovered to stimulate collagen synthesis of mesenchymal stem cells (MSCs) [15]. When KGN bonded with Filamin A (FLNA),

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KGN disrupted FLNA's interaction with the transcription factor core-binding factor beta subunit (CBFβ). This study enlightened the future of small molecule-based approaches to adult stem cell therapies. Specific cell populations with variable mesenchymal differentiation potential were obtained from freshly separated and adherent cultured dermal fibroblasts. Various studies proved the fibroblasts had the capacity to differentiate into multiple cell lineages[16–18]. Given the phenotypic similarity of MSCs and dermal fibroblasts [19–21], we wonder what effect could KGN have on human dermal fibroblasts? As such, we proposed and tested the hypothesis that the small molecule KGN could stimulate collagen synthesis in human dermal fibroblasts in vitro, and then we examined its effect in the dermis of mice. We further investigated the potential biochemical mechanism of how KGN activated collagen expression in fibroblasts. If these mechanisms were observed, the novel small molecule KGN could be applied conveniently in tissue engineering of fibroblasts, accelerating wound healing, or esthetic and reconstructive skin rejuvenation, instead of expansive tissue components (collagen) filling or complex cell transplantation.

2. Materials and methods

All animal procedures were conducted according to the Guide for the Care and Use of Laboratory Animals. All mice were maintained in a pathogen-free environment. All experiments were performed under laminar flow hoods.

2.1. Cells isolation and culture

Human fibroblasts were isolated and cultured as previously described [22]. A humidified 37 °C/5% CO_2 incubator was used for cell culture with basal medium supplemented with 10% FBS (Hyclone, Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin, and 100 mg/L streptomycin (Hyclone, Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Cell viability assay

For the cell viability assay, fibroblasts were seeded on six-well plates, followed by KGN (cat. no. 420358; Calbiochem, Millipore, Billerica, MA, USA) or dimethyl sulfoxide (DMSO; Sigma–Aldrich, St. Louis, MO, USA) treatment. According to the manufacturer's instructions, cell viability and viability were assessed by AlamarBlue® assay (Invitrogen, Carlsbad, CA, USA).

2.3. Phalloidin staining

Formalin fixation (3.5%; Sigma–Aldrich, St. Louis, MO, USA) was performed for 10 min at room temperature. Cells were permeabilized (0.25% Triton X-100; Sigma–Aldrich, St. Louis, MO, USA), and nonspecific binding was blocked (3% bovine serum albumin [BSA]). F-actin was stained with Alexa Fluor 488 Phalloidin for 30 min at room temperature (1:200; Cytoskeleton, Inc., Denver, CO, USA). Cells were visualized using a confocal microscope (Leica, Solms, Germany).

2.4. Real-time reverse transcription polymerase chain reaction (RT-PCR)

The total RNA of cells was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After reverse transcription reaction, real-time polymerase chain reaction (PCR) was performed by Roche480 system using SYBR® Premix (Takara, Dalian, China) according to the manufacturer's instructions. The conditions of real-time PCR were as follows: Denaturation at 95 °C for 10 s, 40 cycles at 95 °C for 10 s, and 60 °C for 30 s. Dissociation stage was added to the end of the amplification procedure. GAPDH was used as internal control. Data were analyzed using the comparison Ct ($2^{-\Delta\Delta Ct}$) method and expressed as fold change compared to the respective control. Each sample was analyzed in triplicate. Primers sequences were as follows: type-I collagen, 5′-GAACGCGTGTCATCCCTTGT-3′ (forward) and 5′-GAACGAGGTAGTCTTTCAGCAACA-3′ (reverse); MMP9, 5′-GGGACGCAGACATCGTCATC-3′ (forward) and 5′-TCGTCA TCGTCGAAATGGGC-3′ (reverse); MMP1, 5′-CTGGCCACAACTGC CAAATG-3′ (forward) and 5′-CTGTCCCTGAACAGCCCAGTACTTA-3′ (reverse); α-sma, 5′-GGCATTCACGAGACCACCTAC-3′ (forward) and 5′-CGACATGACGTTGTTGGCATAC-3′ (reverse); GAPDH, 5′-AGGTCGGTGTGAACGGATTTG-3′ (forward) and 5′-TGTAGAC CATGTAGTTGAGGTCA-3′ (reverse).

2.5. Western blot

For Western blot analysis, Total proteins were extracted from cultured cells using radioimmunoprecipitation assay (RIPA) lysis buffer. Protein concentrations were determined using a bicinchoninic acid (BCA) assay. Thirty micrograms of each protein lysate was resolved using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Following transfer, membranes were blocked and then incubated with primary antibodies overnight at 4 °C.

The primary antibodies used were anti-smads, anti-MAPK family antibodies (Cell Signaling Technology, Beverly, MA, USA) at a dilution of 1:1,000 and anti-collagen I (Abcam, Cambridge, UK) at a dilution of 1:1,000. For normalization of protein loading, GAPDH (Sigma–Aldrich, St. Louis, MO, USA) antibody was used at a dilution of 1:5,000. Immunoreactive bands were quantitatively analyzed in triplicate by normalizing the band intensities to their respective controls on scanned films with ImageJ software.

2.6. Smad5 knockdown by siRNA

RNA interference was performed using smad5 siRNA (h) (sc-38378; Santa Cruz Biotechnology, Dallas, TX, USA), targeting human smad5 and control siRNA (sc-37007) as negative control. Transfection for primary fibroblasts was conducted using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

2.7. Immunofluorescence

After being subjected to KGN for 12 h, fibroblasts were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.25% Triton X-100 for 15 min, and blocked with 3% BSA for 60 min. Anti-smad5 and smad4 at a dilution of 1:200 were used to incubate cells for 2 h at room temperature. After the final wash, the nuclei was counterstained by adding 100 μL of a 2-mg/mL solution of 4′,6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich, St. Louis, MO, USA) in 1× phosphate-buffered saline (PBS) for 10 min before imaging. Cells were visualized using a confocal microscope (Leica, Solms, Germany).

2.8. Histology and Masson's trichrome stain

Tissue specimens were collected and fixed with 4% paraformaldehyde for 24 h and embedded with paraffin. Sections of 6 μm were stained with hematoxylin and eosin for conventional morphological evaluation. As for Masson's trichrome stain, the sections were fixed in preheated bouin's solution at 56 °C for 15 min and

then stained with Masson's Trichrome stain kit (Trichrome stain LG solution, HT10316, Sigma–Aldrich, USA).

2.9. Immunohistochemistry

Skin from the model animals were fixed overnight with 4% paraformaldehyde in PBS and then embedded in paraffin. Tissue sections (10 μm) were deparaffinized in xylene, serially rehydrated in ethanol, and washed with PBS. After blocking nonspecific protein binding with 5% BSA in PBS for 30 min at room temperature, sections were incubated at room temperature with primary antibodies against collagen I (Abcam, Cambridge, UK). The slides were rinsed in PBS and then incubated with secondary antibody according to the manufacturer's protocol.

2.10. Statistical analysis

The data were expressed as means ± SD. Results were analyzed using Student's *t*-test using the SPSS 13.0 software (SPSS Inc., USA). $P < 0.05$ indicated a significant difference between groups.

3. Results

3.1. No obvious cytotoxicity exerted by KGN on fibroblasts' viability, morphology and survival

To study KGN's effect on fibroblasts (Fig. 1A), cells were administered with KGN at the concentrations of 100 nM and 1 μM for 6, 12, 24, and 48 h. The results showed that KGN had no obvious influence on fibroblasts' viability during 48 h (Fig. 1B). In addition, no significant difference in percentages of apoptotic cells was observed (Fig. 1C) after the exposure of cells to KGN. KGN was confirmed to bind to FLNA, an actin-binding protein that cross-links actin filaments. To study KGN's influence on fibroblasts' cytoskeletal network, F-actin was stained with phalloidin after cells were treated with KGN for 24 h. No obvious cell morphological deformation was observed (Fig. 1D). Collectively, KGN has no obvious cytotoxicity on fibroblasts' viability, morphology and survival.

3.2. KGN stimulated collagen synthesis of fibroblasts, but did not induce the expression of α-SMA, MMP1 and MMP9 in vitro

RT-PCR with mRNA isolated from fibroblasts treated with KGN showed that the expression of type-I collagen gene was upregulated in a time-dependent manner, compared with fibroblasts treated with DMSO (Fig. 2A). Changes were observed when fibroblasts were treated with KGN for 12 h (the expression of mRNA was doubled). The upregulation effect remained when fibroblasts were treated with KGN up to 48 h and 96 h. By Western blot analysis, more type-I collagen proteins were examined in KGN-treated fibroblasts than DMSO-treated fibroblasts at 24 and 48 h (Fig. 2B).

These upregulation results of collagen expression were then confirmed by the outcome of the hydroxyproline experiment (Fig. 2C). As a metabolite of collagen, hydroxyproline represents the production of collagen. For the detection of hydroxyproline, an enzyme-linked immunoabsorbent assay (ELISA) was applied. When dermal fibroblasts were treated with DMSO for 24 and 48 h, the expression of hydroxyproline was approximately 0.3 μg/μL. By contrast, when treated with 100 nM or 1 μM KGN, the expression of hydroxyproline significantly increased to 0.7 μg/μL.

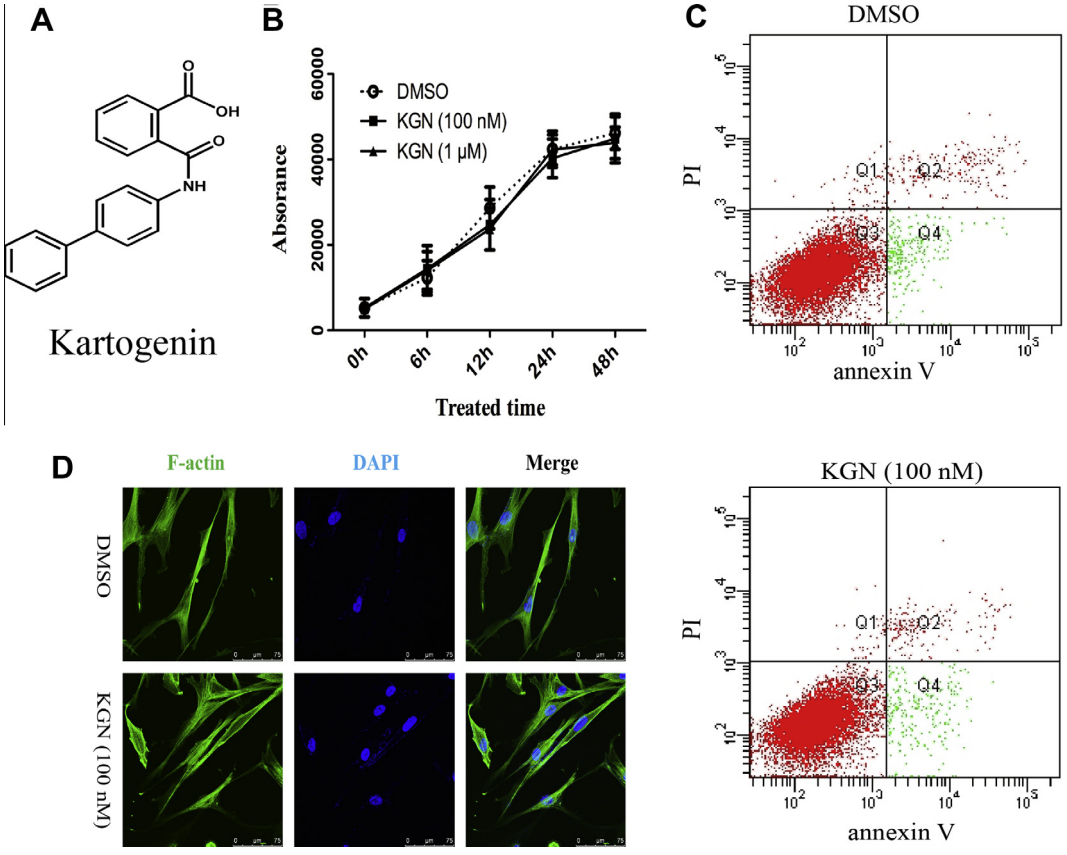

Fig. 1. KGN had no influence on fibroblasts' morphology, survival, and viability. (A) The chemical structure of KGN is shown. (B) Cell viability was examined by AlamarBlue test at 6, 12, 24, and 48 h after KGN was applied at concentrations of 100 nM, and 1 μM. (C) After treatment with KGN and DMSO, apoptosis was analyzed and examined by PI–annexin V fluorescence by fluorescence-activated cell sorting analysis. (D) The effect of KGN on the cytoskeleton was studied by F-actin staining by phalloidin. Data were presented as the mean ± standard deviation (SD), $n \geqslant 3$.

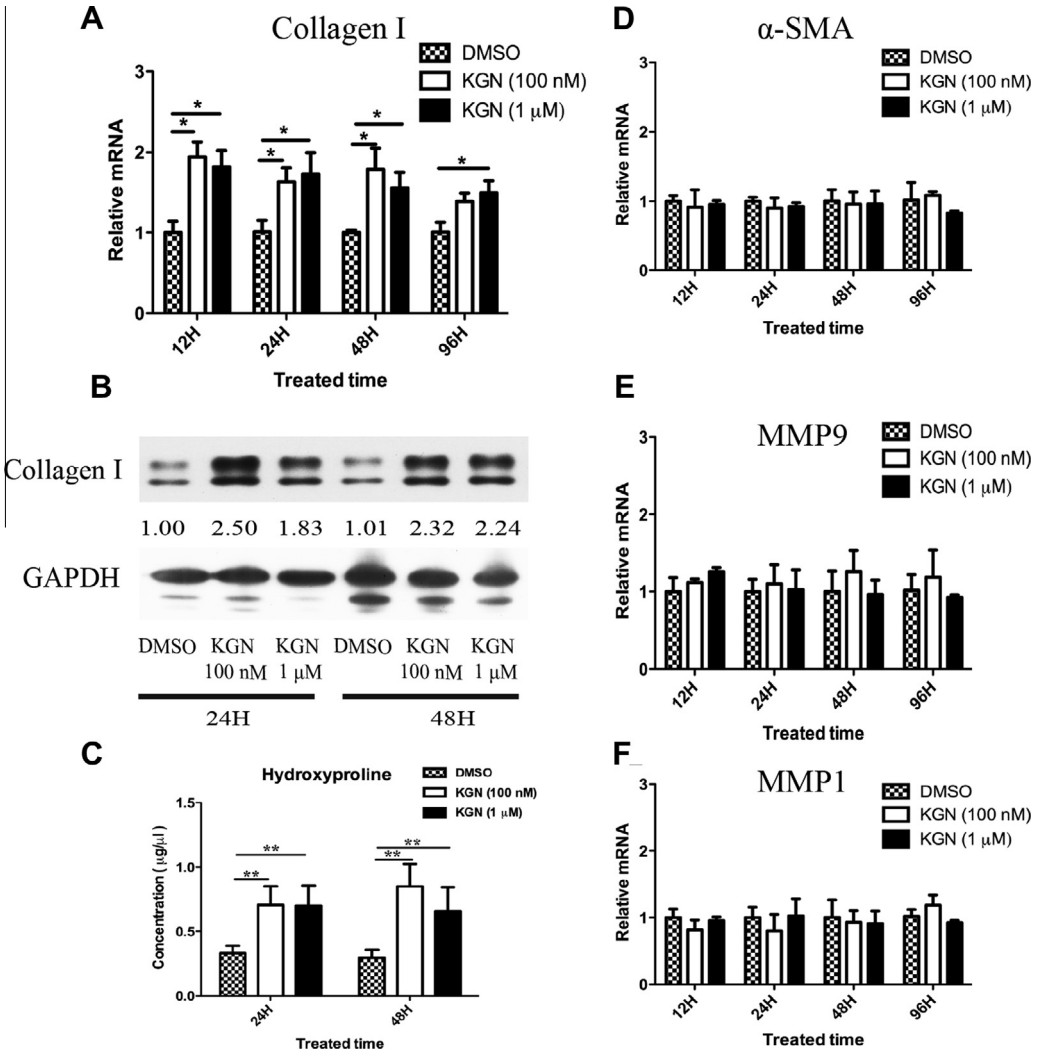

Fig. 2. KGN stimulated collagen synthesis of fibroblasts. The relative expression of type-I collagen (A) was measured by real-time PCR at 12, 24, 48, and 96 h after KGN was applied at concentrations of 100 nM, and 1 μM. GAPDH expression was used as an internal control. (B) The protein expression of type-I collagen was also assessed by Western blot at 24 and 48 h after KGN was applied at concentrations of 100 nM, and 1 μM. (C) Hydroxyproline was measured by an enzyme-linked immunoabsorbent assay (ELISA) kit. (D–F) The expression of α-sma, MMP9 and MMP1 were measured by real-time PCR. Data were presented as the mean ± SD, $n \geqslant 3$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Moreover, the expression of α-SMA, which was implicated in fibrosis by extracellular collagen fiber deposition and MMP1 and MMP9, well-known proteases that degrade collagen proteins, were unchanged when fibroblasts were treated with KGN (Fig. 2D–F).

3.3. Smad4/smad5 pathway was activated by KGN

Next, the potential biochemical mechanism of how KGN activated collagen I gene expression was investigated. We focused on the down-signaling pathway of TGF-β and MAP kinase pathway. As shown in Fig. 3A, when fibroblasts were treated for 12 and 24 h, KGN (100 nM and 1 μM) markedly increased the expression of phosphorylated smad5 protein compared with DMSO. However, phosphorylated and total smad2, total protein level of smad4, phosphorylated and total smad3 protein all remained unaltered. The band intensity of p-smad5, p-smad2 and p-smad3 were quantified (Fig. 3B). Previous studies also revealed that MAP kinase pathway play roles in peptide induced collagen synthesis of fibroblasts[23]. In order to elucidate the underlying mechanism of positive effect of KGN on collagen production, MAP kinase signaling pathways were also investigated. However, as shown in Fig. 3C, when fibroblasts were treated for 12 and 24 h with KGN (100 nM and 1 μM), the expression of total or phosphorylated ERK, JNK and p38 protein remained unchanged, compared with DMSO.

Furthermore, once smad5 was inhibited by specific siRNA for smad5 (Fig. 3D), the upregulation effect of type-I collagen protein expression was obviously decreased, which confirmed the role of smad5 in the transduction of the KGN effect. Immunofluorescence experiments further demonstrated that KGN activated the smad4/smad5 pathway, but not smad2/smad3 (Fig. 3E). After treatment with KGN at the concentration of 100 nM for 12 h, p-smad5 protein (labeled by red) was increased and completely translocated into the nucleus (labeled by blue). Smad4 protein (labeled by green) was also enhanced and mostly translocated into the nucleus. By contrast, in the DMSO groups, both p-smad5 and smad4 were retained in the cytoplasm. Smad2/smad3 and p-smad2/p-smad3 remained in the cytoplasm (not shown in the figure).

3.4. KGN stimulated collagen synthesis in the mouse dermis

To validate this phenomenon further, we examined the effect of KGN on type-I collagen expression in dermal cells in situ by HE, Masson's trichrome stain and immunohistochemistry. KGN at the concentration of 100 nM was delivered by microneedles (MTS-Roller Model: CR2 (0.2 mm)) to the dermis of normal BALB/C

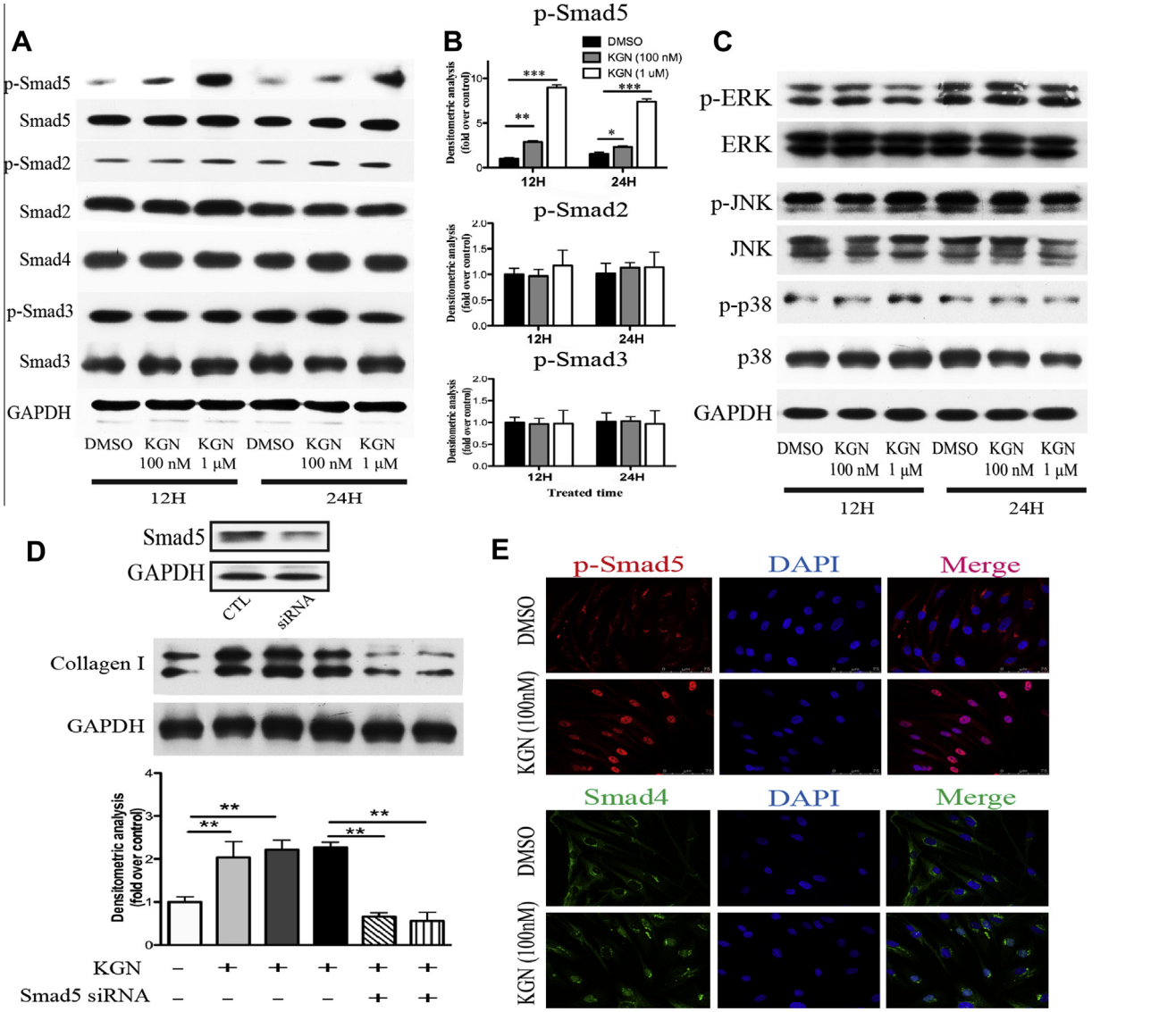

Fig. 3. Smad5 functioned as a downstream effector of KGN, but not smad2, smad3, or MAPK signaling pathway. (A–C) Western blot showed the expression of phosphorylated and total smad5, smad2, smad3, smad4, ERK, JNK and p-38 in fibroblasts with or without KGN stimulation for 12 or 24 h. (B) The band intensity corresponding to phosphorylated smad2, smad3 and smad5 were quantified and normalized to GAPDH using imageJ. After confirming the efficiency of siRNA for smad5 (D), Western blot showed the expression of type-I collagen in fibroblasts when cells were treated with DMSO (as negative control), KGN or KGN with specific siRNA of smad5. GAPDH expression was used as internal loading control. (E) Immunofluorescence experiments: p-smad5 was labeled as red and smad4 was labeled as green. Nucleus (blue) was stained with DAPI. Data were presented as the mean ± SD, $n \geqslant 3$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mice of 8 weeks, compared with DMSO. Each group included 6 mice. Microneedles ensured the needed depth and penetration of skin's stratum corneum, for KGN to dissolve in dermal layer[24]. Results showed that, whereas collagens were dispersed in DMSO-treated dermis of BALB/c mice, dermis in the KGN (100 nM)-treated group exhibited increased dermal thickness and intense blue staining (Fig. 4A–D), which represented more collagen composition in the dermis. Immunohistochemistry using anti-collagen I antibodies (Fig. 4E) showed compact and clearly evident staining in the KGN (100 nM)-treated dermis in all sections, compared to loosely distributed collagen I in the DMSO-treated group.

4. Discussion

Declined production of collagen by fibroblast is one of the major causes of wrinkles development and aging appearance. The plant-derived compounds were widely used in the cosmetic market. However, most natural compounds found in cosmetic products were attributed to their anti-oxidative properties, only few of them were able to directly increase collagen synthesis. Ginsenoside Rb_1 (molecular weight: 1109), the major ginsenoside in *Panax ginseng*, was shown to induce type-I collagen expression in dermal fibroblasts[25,26]. Another study found that a laminin tyrosine–isoleucine–glycine–serine–arginine (YIGSR) peptide could enhanced collagen synthesis of human dermal fibroblasts[23]. However, these two macromolecular compounds could not easily penetrate the epidermis and reach fibroblasts in the deep skin tissue. In this study, we found that a novel small heterocyclic molecule KGN (molecular weight: 317.3) could induce the collagen synthesis of human dermal fibroblasts. This lipophilic molecule could be conveniently used locally on the skin without the application of transdermal drug delivery systems.

This cell-permeable compound KGN reversibly binds to the FC-1 fragment of filamin A and disrupts its association with CBFβ leading to nuclear localization of CBFβ[15]. Aya Sasaki [27]

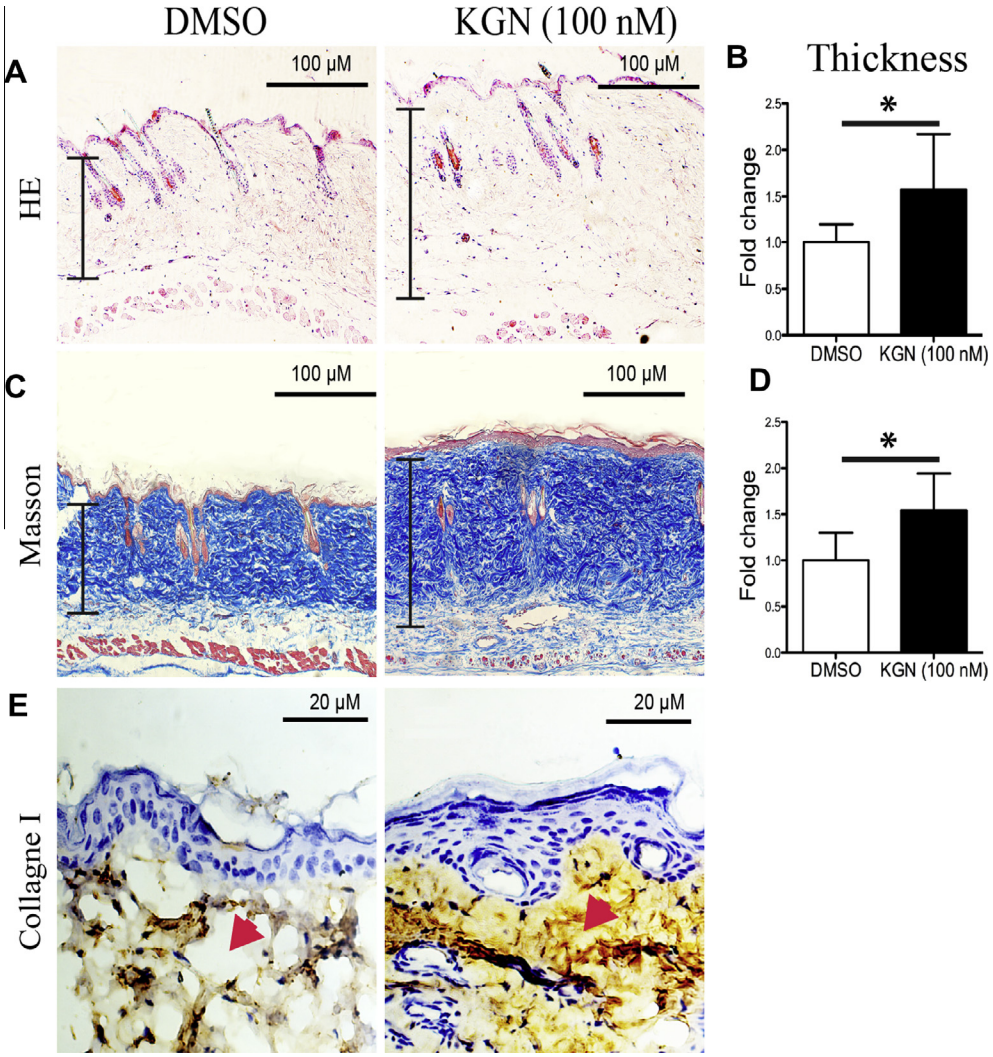

Fig. 4. The effect of KGN on the dermis of mice. KGN was delivered into the dermis of normal BALB/c mice of 8 weeks. (A) HE stained dermis of DMSO and KGN (100 nM) treated mice. (B) The thickness of dermis from 6 KGN-treated mice was analyzed and quantified, compared to DMSO-treated group. (C–D) Masson's trichrome stain showed collagen density in the dermis, and the thickness of blue-stained dermis was analyzed. (E) Immunohistochemistry of dermis of mice, using anti-collagen I monoclonal antibody (brown staining; red arrow) between the KGN-treated and DMSO-treated dermis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

discovered the interaction of filamin A with smad5. Smad5 protein directly binds to FC-1 fragment of filamin A. Given the same binding site to the filamin A between smad5 and CBFβ, we investigated the effect of KGN on the smad5 signaling pathway, which was confirmed by our results. In this study, we found a novel signaling pathway was induced by KGN that transcriptional factor smad5 was activated and translocated into the nuclear. KGN markedly increased the expression of phosphorylated smad5 protein while phosphorylated and total smad2, phosphorylated and total smad3 protein all remained unaltered. Complex signaling pathways are involved in the regulation of collagen expression. Smads interact with numerous transcription factors [28,29] such as CBF. Three mammalian α subunits of CBF form complexes with smad5 [30]. As such, we expect that, when CBFβ and smad5 are disassociated from FLNA by KGN, a complex transcriptional regulation network may form between the aforementioned transcriptional factors, which demand further elaborate investigation.

This small molecule KGN may be applicable in tissue engineering, wound healing, and esthetic purposes. A decrease in the production of collagen by aged fibroblasts, which accounts for the overall 20% decrease in dermal thickness, was observed. Cosmetic collagen or hyaluronic acid injection has become the most popular esthetic surgery for facial rejuvenation. Compared to the complex and expansive collagen or hyaluronic acid injection procedure, KGN could be freely used to stimulate the endogenous collagen production of the patients' own fibroblasts, without issues of collagen source and immunogenicity. As for tissue engineering, fibroblasts were used as one of the easiest cell sources. The dermal fibroblasts exist in large numbers throughout the connective tissue in the adult human and primary cultures can be obtained through minimally invasive biopsies[31]. Complicated methods had been exploited to induce osteogenic, adipogenic, and chondrogenic differentiations of fibroblasts in vitro. KGN could be added into the chondrogenic differentiation medium of fibroblasts. For wound healing, KGN may also be applied, without obvious cytotoxicity, to improve this process when amounts of collagen were secreted by dermal fibroblasts and deposited [32].

Our study showed that a novel small heterocyclic molecule KGN could disassociate smad5 into the nucleus, inducing the collagen synthesis of human dermal fibroblasts. This molecule could be used in tissue engineering of fibroblasts, wound healing, or esthetic and reconstructive purpose of rejuvenation. Although more elaborate investigations are needed to elucidate the potential complex

transcriptional regulation network of CBF and smads family, our results intensify the future of small molecule-based approaches to endogenous cells manipulation [33].

Acknowledgments

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