Original Research

Inhibitory effect of ginsenoside Rg1 on extracellular matrix production via extracellular signal-regulated protein kinase/activator protein 1 pathway in nasal polyp-derived fibroblasts

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Abstract

Nasal polyps are associated with chronic inflammation of the sinonasal mucosa and are involved in myofibroblast differentiation and extracellular matrix (ECM) accumulation. Ginsenoside Rg1, a compound derived from *Panax ginseng*, shows antifibrotic and anticancer effects. However, the molecular effects of Rg1 on myofibroblast differentiation and ECM production remain unknown. The aims of this study were to investigate the effect of Rg1 on transforming growth factor (TGF)-β1-induced myofibroblast differentiation and ECM production and to determine the molecular mechanism of Rg1 in nasal polyp-derived fibroblasts (NPDFs). NPDFs were isolated from nasal polyps of seven patients who had chronic rhinosinusitis with nasal polyp. NPDFs were exposed to TGF-β1 with or without Rg1. Expression levels of α-smooth muscle actin (SMA), fibronectin and collagen type Iα1 were determined by reverse transcription polymerase chain reaction, Western blot and immunofluorescent staining. TGF-β1 signaling molecules, including Smad2/3, extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 were analyzed by Western blotting. Transcription factors involved with TGF-β1 signaling, nuclear factor (NF)-κB and activator protein 1 (AP-1) were also assessed by Western blot. The cytotoxic effect of Rg1 was measured by an established viability assay. The mRNA and protein expression levels of α-SMA, fibronectin and collagen type Iα1 were increased in TGF-β1-induced NPDFs. Rg1 inhibited these effects. The inhibitory molecular mechanism of Rg1 was involved in the ERK pathway. Rg1 inhibited the transcription factor activation of AP-1. Rg1 itself was not cytotoxic. The ginsenoside Rg1 has inhibitory effects on myofibroblast differentiation and ECM production. The inhibitory mechanism of Rg1 is involved with the ERK and AP-1 signaling pathways. Rg1 may be useful as an inhibitor of ECM deposition, and has potential to be used as a novel treatment option for nasal polyps.

Keywords: nasal polyp, myofibroblasts, extracellular matrix, TGF-β1, ginsenoside Rg1, Smad2/3, MAPK, NF-κB, AP-1

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Introduction

Nasal polyps are a chronic inflammatory condition of the paranasal sinuses; their overall prevalence ranges from 1% to 4% in the general population.[1] The typical histological characteristics of nasal polyps include edematous fluid with sparse fibrous cells, few mucous glands without innervation, proliferation of stromal and epithelial elements, and a thickening of the basement membrane.[1,2] Although the etiologies of nasal polyps and the pathophysiological mechanisms leading to nasal polyp formation are still poorly understood, evidence suggests that damage to the mucosal epithelium is accompanied by extracelluar matrix (ECM) accumulation and inflammatory cell infiltration, which play an important role in nasal polyp formation.[3]

Fibroblasts, which are found in the stroma and are the cellular source of ECM proteins, are involved in the nasal polyp growth process.[4] Among the fibroblasts, myofibroblasts are an activated cell phenotype with tissue contractile properties and a high capacity for ECM protein secretion. A major process in the development of nasal polyps is the ECM accumulation produced by differentiation of fibroblasts into myofibroblasts.[4]

Transforming growth factor (TGF)-β1 modulates cell activation, proliferation, differentiation, wound healing and angiogenesis. Among its many activities, TGF-β1 induces myofibroblast differentiation and ECM accumulation in nasal polyps.[5] Myofibroblasts express α-smooth muscle actin (α-SMA) and produce large amounts of ECM components. TGF-β1 has also been shown to be involved in the pathogenesis of chronic upper airway disorders such as nasal polyps and chronic rhinosinusitis.[6]

Ginseng, the root of *Panax ginseng* C.A. Meyer, Family Araliaceae, is frequently used as a crude substance in Asian countries as a food product as well as a medicinal ingredient. Ginseng roots are used clinically for various diseases, including atherosclerosis, liver dysfunction, cerebrovascular diseases, skin inflammation and postmenopausal disorder.[7,8] Most pharmacological actions of ginseng are attributed to ginsenosides, which are major components extracted from different species of ginseng. Rg1, a panaxatriol saponin, is one of the major active molecules isolated from ginsenosides.[7,8] Rg1 has been shown to be capable of suppressing liver ECM production in a rat model, likely by inhibition of stellate cell activity.[9] Rg1 can significantly attenuate the development of myocardial fibrosis and has an antiproliferative effect in human arterial smooth muscle cells.[10] Rg1 restrains renal tubular epithelial cell to myofibroblast transition *in vitro*.[11] However, the inhibitory role of ginsenoside Rg1 in myofibroblast differentiation and ECM accumulation remains to be clarified, and the molecular mechanisms underlying the inhibitory effects of Rg1 are still not clear.

This study investigated the effect of Rg1 on TGF-β1-induced myofibroblast differentiation and ECM production, and determined the molecular mechanism of Rg1 in nasal polyp-derived fibroblasts (NPDFs).

Materials and methods

Reagents

Human recombinant TGF-β1 was obtained from R&D Systems (Minneapolis, MN, USA). Ginsenoside Rg1 (NPC Biotechnology, Daejeon, Korea) and U0126 (Calbiochem, San Diego, CA, USA), a specific extracellular signal-regulated protein kinase (ERK) inhibitor, were dissolved in dimethylsulfoxide (DMSO) and then diluted to the desired concentrations with complete medium for use in the experiments. The final concentration of DMSO was $<0.1\%$ (v/v) of total medium volume and did not contribute to toxicity.

Nasal polyp tissues

Seven patients with nasal polyps were recruited from the Department of Otorhinolaryngology, Korea University Medical Center, Korea. Informed consent was obtained from each patient, and the study was approved by the Korea University Medical Center Institutional Review Board (KUGGR-2010-052). Nasal polyp tissues were obtained from the region of the middle meatus at the beginning of the endoscopic surgical procedure. All patients had no history of allergy, asthma or aspirin sensitivity.

NPDF culture

NPDFs were isolated from surgical tissues by enzymatic digestion with collagenase (500 U/mL; Sigma-Aldrich, St Louis, MO, USA), hyaluronidase (30 U/mL; Sigma-Aldrich) and DNase (10 U/mL; Sigma-Aldrich). Cells were cultured in Dulbecco's modified Eagles medium containing 10% (v/v) heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 1% (v/v) 10,000 U/mL penicillin and 10,000 μg/mL streptomycin (Invitrogen). The purity of obtained NPDFs was confirmed by observations of their characteristic spindle-shaped cell morphology and by fluorescence-activated cell sorting (FACS). Approximately 95% of cells in cultured NPDFs were positive for vimentin and Thy-1, which were used as fibroblast markers, but negative for epithelial cell markers such as E-cadherin. Experimental cells were obtained from the fourth cell passage.

Reverse transcription polymerase chain reaction

NPDFs were exposed to Rg1 (5–40 ng/mL) with or without TGF-β1 (5 ng/mL) for 24 h. Total RNA was isolated according to the manufacturer's recommendations using Trizol reagent (Invitrogen). Two micrograms of RNA were reverse-transcribed using MMLV reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was performed using the following primers: *α-SMA* (sense sequence 5′ GGTGCTGTCTCTCTATGCCT CTGGA 3′, antisense sequence 5′ CCCATCAGGCAAC TCGATACTCTTC 3′, 322 bp), *fibronectin* (sense sequence 5′ GGATGCTCCTGCTGTCAC 3′, antisense sequence 5′ CTGTTTGATCTGGACCTGCAG 3′, 382 bp), *collagen type Iα1* (sense sequence 5′ CATCACCTACCACTGCAAGAA C 3′, antisense sequence 5′ ACGTCGAAGCCGAATTCC 3′, 278 bp) and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*; sense sequence 5′ GTGGATATTGTTGCCATCAA TGACC 3′, antisense sequence 5′ GCCCCAGCCTTCTTCAT GGTGGT 3′, 271 bp). The gels were captured and visualized using a ChemiDoc XRS + molecular imager (Bio-Rad, Hercules, CA, USA).

Western blot analysis

NPDFs were exposed to Rg1 with or without TGF-β1. Time for treatment was 24 h for α-SMA, 48 h for fibronectin and collagen type Iα1, 30 min for pERK, p-p38 and pJNK (JNK, c-Jun N-terminal kinase), and 60 min for pSmad2/3, p50 and c-Fos detection. Lysates of NPDFs were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the resolved proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk solution and were incubated with the following antibodies: α-SMA (Chemicon, Billerica, MA, USA), fibronectin, collagen type Iα1, pSmad2/3, pJNK, p50, c-Fos, lamin A/C, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), pERK, ERK, p-p38 and p38 (Cell Signaling Technology, Beverly, MA, USA). Blots were visualized with the application of horseradish peroxidase-conjugated secondary antibody followed by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

Immunofluorescence staining

NPDFs were exposed to Rg1 with or without TGF-β1 for 24 h for α-SMA and 48 h for fibronectin and collagen type Iα1 detection. NPDFs were fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100 in 1% bovine serum albumin for 10 min, blocked with 5% bovine serum albumin for one hour at room temperature and incubated overnight at 4°C with anti-α-SMA antibody (Chemicon), anti-fibronectin or anti-collagen type Iα1 antibody (Santa Cruz Biotechnology). NPDFs were then incubated with goat anti-mouse Alexa 488 (Invitrogen) or goat anti-rabbit Alexa 555 (Invitrogen) secondary antibodies. Stained NPDFs were captured and visualized using a LSM700 confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

Statistical analysis

The results were obtained from at least three independent experiments. Results are presented as the mean $\pm$ SEM. The statistical significance of the differences between control and experimental data was analyzed using one-way analysis of variance followed by Tukey's test (GraphPad Prism, version 5; GraphPad Software, San Diego, CA, USA). Significance was established at the 95% confidence level. P values <0.05 were accepted as statistically significant.

Results

Effect of Rg1 on myofibroblast differentiation and ECM production in TGF-β1-induced NPDFs

TGF-β1 is a potent stimulus for the differentiation of fibroblasts into myofibroblasts in various organs such as the kidney, lung and the nasal passage.[4,12,13] To investigate the effect of Rg1 on myofibroblast differentiation in TGF-β1-induced NPDFs, NPDFs were treated with TGF-β1 with or without Rg1 and were analyzed for α-SMA, a marker of myofibroblast differentiation, and ECM expression including fibronectin and collagen type Iα1. Expression levels of *α-SMA*, *fibronectin* and *collagen type Iα1* mRNA were determined by reverse transcription (RT)-PCR (Figure 1a). All three mRNA levels were increased after TGF-β1 stimulation, and then significantly decreased in Rg1 treatment in a dose-dependent manner. The relative mRNA expression levels were quantified (Figure 1b). Protein expression levels were demonstrated by Western blot (Figure 1c) and immunofluorescence staining (Figure 1d). α-SMA, fibronectin and collagen type I protein levels increased in TGF-β1-treated NPDFs, and then Rg1 significantly reduced the induced expression of these proteins in a dose-dependent manner. These results indicate that Rg1 inhibits TGF-β1-induced myofibroblast differentiation and ECM production in NPDFs.

Effect of Rg1 on pSmad2/3 and MPAK in TGF-β1-induced NPDFs

TGF-β activates phosphorylation of Smad2/3, and then phosphorylated Smad2/3 induces myofibroblast differentiation and ECM production.[14,15] To identify whether TGF-β1 induces phosphorylation of Smad2/3 and Rg1 inhibits the activation of Smad2/3, we investigated phosphorylated Smad2/3 by Western blot in NPDF cells (Figure 2a). Activation of Smad2/3 was identified using an antibody specific for the phosphorylated Smad2/3 (p-Smad2/3). TGF-β activated phosphorylation of Smad2/3. However, Rg1 did not inhibit the activation of Smad2/3. To determine another inhibitory molecular mechanism involved in Rg1 after TGF-β1 stimulation in NPDFs, we investigated the mitogen-activated protein kinase (MAPK) signaling pathways including ERK, p38 and JNK. Activation of ERK, p38 and JNK was identified using antibodies specific for the phosphorylated form of ERK (pERK), p38 (p-p38) and JNK (pJNK) by Western blot (Figure 2b). TGF-β1 induced MAPK signaling pathways including ERK1/2, p38 and JNK phosphorylation. However, Rg1 inhibited only ERK1/2 activation among the three MAPK signaling pathways.

Effect of Rg1 on transcription factor in TGF-β1-induced NPDFs

To investigate the activation of transcription factor by TGF-β1, we investigated nuclear factor-kappa B (NF-κB) and activator protein 1 (AP-1) expression levels by Western blot analysis (Figure 3). Activation of NF-κB was identified using p50 antibody, which is a subunit of NF-κB, and activation of AP-1 was identified using c-Fos antibody, which is a subunit of AP-1. TGF-β1 activated both NF-κB and AP-1. However, Rg1 inhibited only AP-1 activation, not NF-κB.

Inhibitory mechanism of Rg1 on myofibroblast differentiation and ECM production via ERK1/2 pathway

To evaluate the inhibitory mechanism by Rg1, NPDFs were treated with Rg1 or U0126, a specific inhibitor of MAPK/ERK kinase, and determined by Western blot analysis. Activation of ERK1/2 by TGF-β1 was attenuated by treatment of U0126 as well as Rg1 (Figure 4a). TGF-β1-induced c-Fos activation was significantly suppressed by treatment with Rg1 or U0126 (Figure 4b). To assess whether inhibition by U0126 influences myofibroblast differentiation and ECM production, we assessed α-SMA, fibronectin and collagen type Iα1 protein expression levels after treatment with U0126 or Rg1 (Figure 4c). Western blot analysis showed that protein expression levels of α-SMA, fibronectin and collagen type Iα1 were markedly inhibited by treatment with U0126 or Rg1 in TGF-β1-induced NPDFs.

Discussion

Nasal polyps are part of a chronic inflammatory disease that involves benign outgrowths from the mucous membranes of the nasal cavity or paranasal sinuses. The condition is characterized by edematous masses of inflamed mucosa prolapsing into the nose, leading to nasal obstruction,

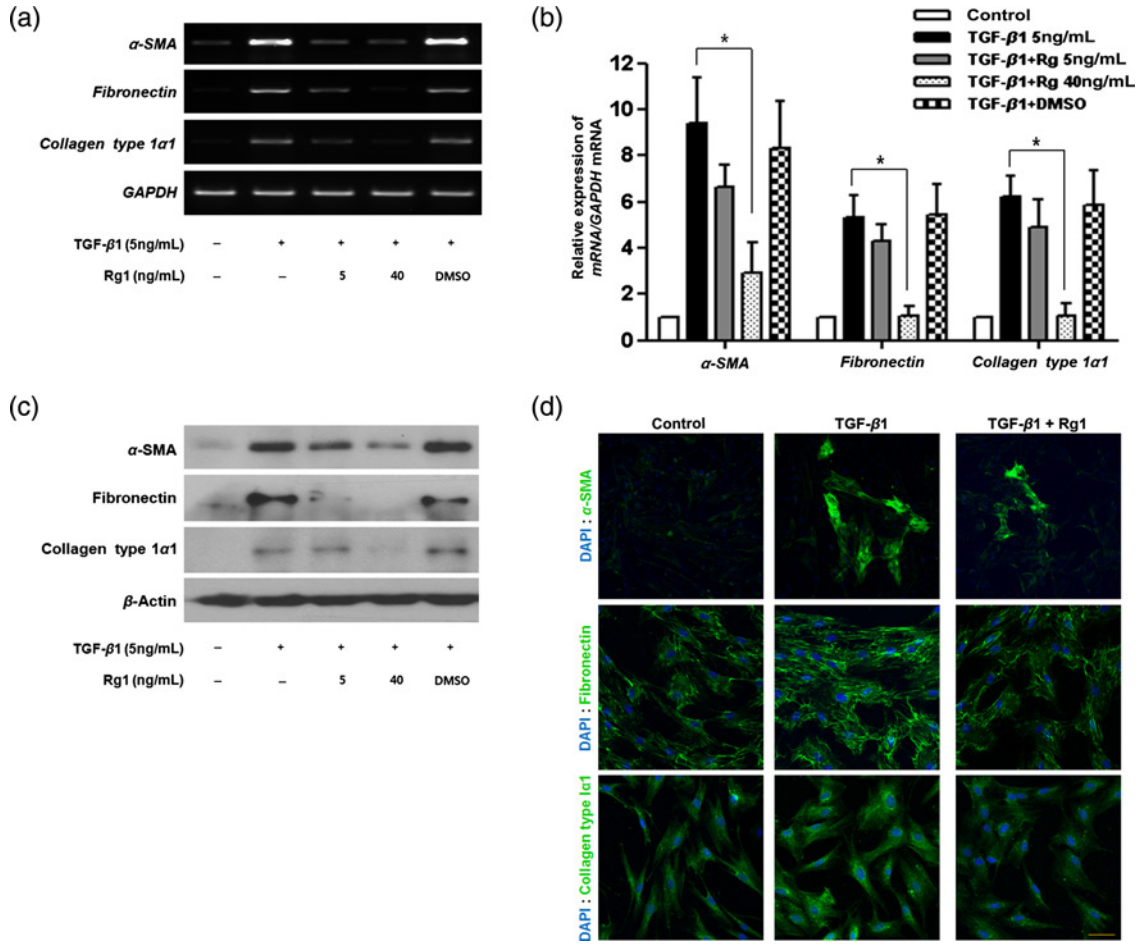

Figure 1 Effect of Rg1 on myofibroblast differentiation and extracelluar matrix production in nasal polyp-derived fibroblasts (NPDFs). NPDFs induced by TGF-β1 were treated with or without Rg1 (5 or 40 ng/mL). (a) The expression levels of mRNA including α-*SMA*, *fibronectin* and *collagen type I*α*1* were determined by semi-quantitative RT-PCR. The expression of *GAPDH* is shown as an internal control. (b) Quantification of mRNA expression levels. The results are obtained from at least three independent experiments. Values are means $\pm$ SEM. $^{*}P$ value <0.05. (c) The expression levels of protein including α-SMA, fibronectin and collagen type I were determined by Western blot. The expression of β-actin is shown as an internal control. (d) Localization and expression of α-SMA, fibronectin and collagen type Iα1 proteins by immunofluorescent staining. The nucleus was counterstained using 4′,6-diamidino-2-phenylindole (DAPI, in blue). Images were captured and visualized by confocal laser scanning microscopy. The scale bar is 100 μm. α-SMA, α-smooth muscle actin; RT-PCR, reverse transcriptase polymerase chain reaction; TGF-β1, transforming growth factor-β1, GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (A color version of this figure is available in the onine journal)

secretion, loss of smell, headache and reduced quality of life.[1] A major process in the development of nasal polyps is the ECM accumulation produced by differentiation of fibroblasts into myofibroblasts.[3,4] It has also been demonstrated that the number of myofibroblasts observed in nasal polyps is induced by TGF-β1.[5,6] In spite of considerable effort to identify the important factors underlying nasal polyp formation, the etiologies of nasal polyp and the pathophysiological mechanisms for nasal polyp formation are still poorly understood.

Ginseng roots have been frequently used as a food product as well as medicinal ingredient for thousands of years in Asia. Ginsenosides, which are steroidal saponins, are the principal pharmacologically active components of ginseng. Ginsenosides are used clinically for various human diseases, including liver dysfunction, cerebrovascular and cardiovascular diseases, diabetes, obesity and cancers.[7,8,16–18]

In a previous study, ginsenoside Rg1 significantly attenuated the development of myocardial fibrosis in a rat model.[19] In human arterial smooth muscle cells, ginsenoside Rg1 significantly inhibited cell proliferation induced by TNF-α and its antiproliferative effect involved in the inhibition of ERK and PI3K/PKB activation.[10] Ginsenoside Rg1 restrained renal tubular epithelial cell (NRK-52E) to myofibroblast transition *in vitro*.[11] However, it is still not known whether ginsenoside Rg1 inhibits myofibroblast differentiation and ECM production in nasal polyp development.

In this study, we have shown that the ginsenoside Rg1 has an inhibitory effect on TGF-β1-induced myofibroblast differentiation and ECM production, and Rg1 inhibits the ERK1/2 and AP-1 pathways in NPDFs.

TGF-β1 induces α-SMA transcription and myofibroblast differentiation. The activated myofibroblasts express ECM including fibronectin, collagen, elastin and laminin.[4,5,20] TGF-β1 stimulation increased both mRNA and protein expression levels of α-SMA, fibronectin, and collagen type Iα1. On the other hand, ginsenoside Rg1 prevented the effect of TGF-β1 in a dose-dependent manner. These

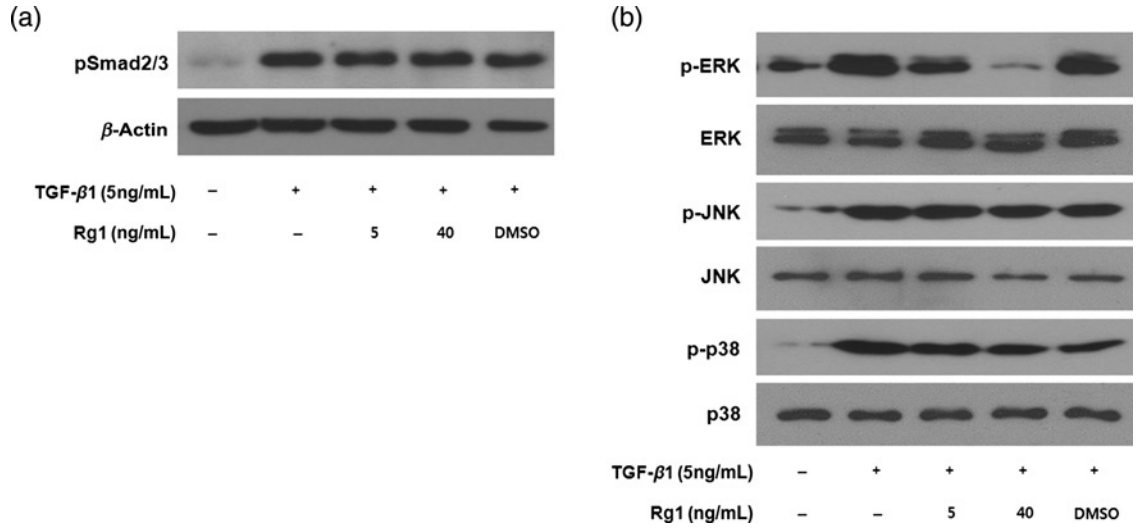

Figure 2 Effect of Rg1 on activation of Smad2/3 and MAPK in nasal polyp-derived fibroblasts (NPDFs). NPDFs induced by TGF-β1 were treated with or without Rg1 (5 or 40 ng/mL). TGF-β1 activates phosphorylation of Smad2/3 and MAPKs including ERK, JNK and p38. (a) Activation of Smad2/3 was determined by Western blot. (b) Western blot shows expression levels of activation of MAPKs such as pERK, pJNK and p-p38. The expression of ERK, JNK, p38 and β-actin are shown as an internal control. TGF-β1, transforming growth factor-β1; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; p-ERK, phosphorylated ERK; p-JNK, phosphorylated JNK; p-p38, phosphorylated p38; DMSO, dimethylsulfoxide

results indicate that Rg1 inhibits TGF-β1-induced myofibroblast differentiation and ECM production in NPDFs.

In fibroblasts, TGF-β1 regulation of α-SMA transcription and myofibroblast differentiation is mediated via TGF-β1 receptor type I phosphorylation of Smad2/3 that subsequently complexes with Smad4 and translocates to the nucleus, where the dimer binds to the promoter region of the α-SMA gene. We investigated whether TGF-β1 activated phosphorylation of Smad2/3. Western blot analyses revealed that Rg1 did not inhibit the activation of Smad2/3. These results indicate that the inhibitory mechanism of Rg1 on TGF-β1-induced myofibroblast differentiation and ECM production does not involve the Smad pathway in NPDFs.

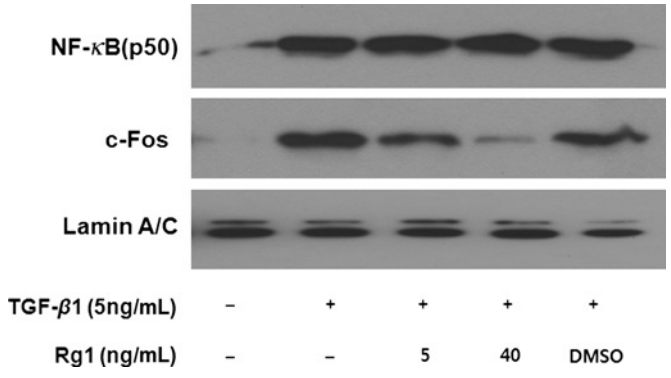

Figure 3 Effect of Rg1 on transcription factor in nasal polyp-derived fibroblasts (NPDFs). NPDFs induced by TGF-β1 were treated with or without Rg1 (5 or 40 ng/mL). Nucleus extracts were isolated and investigated. TGF-β1 activates transcription factors such as NF-κB and AP-1. Expression levels of p50, a subunit of NF-κB, and c-Fos, a subunit of AP-1, were determined by Western blot. The expression of lamin A/C is shown as an internal control in nuclear extracts. TGF-β1, transforming growth factor-β1; NF-κB, nuclear factor-kappa B; AP-1, activator protein 1; DMSO, dimethylsulfoxide

TGF-β1 signaling of tubular epithelial cells in epithelial-to-mesenchymal transition may involve both Smad-dependent[20] and Smad-independent signal transduction pathways such as ERK.[21] Also, ERK acts as an alternative pathway in TGF-β1 signaling of ECM proteins.[12] We demonstrated that TGF-β1 activated MAPK signaling pathways, including ERK1/2, p38 and JNK phosphorylation. However, Rg1 inhibited only ERK1/2 activation among the TGF-β1-induced MAPK signaling pathways. These results indicate that Rg1 inhibits the TGF-β1-induced signal pathway through ERK1/2 in NPDFs.

TGF-β1 participates in early activation of NF-κB DNA binding, which is dependent on TβR-I activation, and correlates with nuclear translocation of the RelA/p65 subunit in human arterial smooth muscle cells.[22] It is believed that AP-1 is a downstream target of MAPK cascades (ERK, JNK and p38 kinase).[13] MAPK signal transduction pathways are involved in the regulation of AP-1-mediated gene expression. Activation of ERK, not p38 kinase, is required for AP-1 DNA binding in TGF-β1-stimulated HLF-02 cells, and increased AP-1 activity regulates myofibroblast differentiation induced by TGF-β1 in lung.[13] In the current study, TGF-β1 activated both NFκB and AP-1. However, Rg1 inhibited only AP-1 activation, not NF-κB. These results indicate that TGF-β1 regulates the DNA binding activity of AP-1 and NF-κB in NPDFs and Rg1 prevents AP-1 activation, but not NF-κB, in TGF-β1-induced myofibroblast differentiation and ECM production. We documented that treatments with Rg1 or U0126, a specific inhibitor of MAPK/ERK kinase, suppressed ERK1/2 and AP-1 activation as well as α-SMA, fibronectin and collagen type Iα1 protein expression levels in TGF-β1-induced NPDFs. These results suggest that TGF-β1 activates ERK1/2 and AP-1, and that AP-1 is the downstream effector of ERK1/2 in TGF-β1-induced

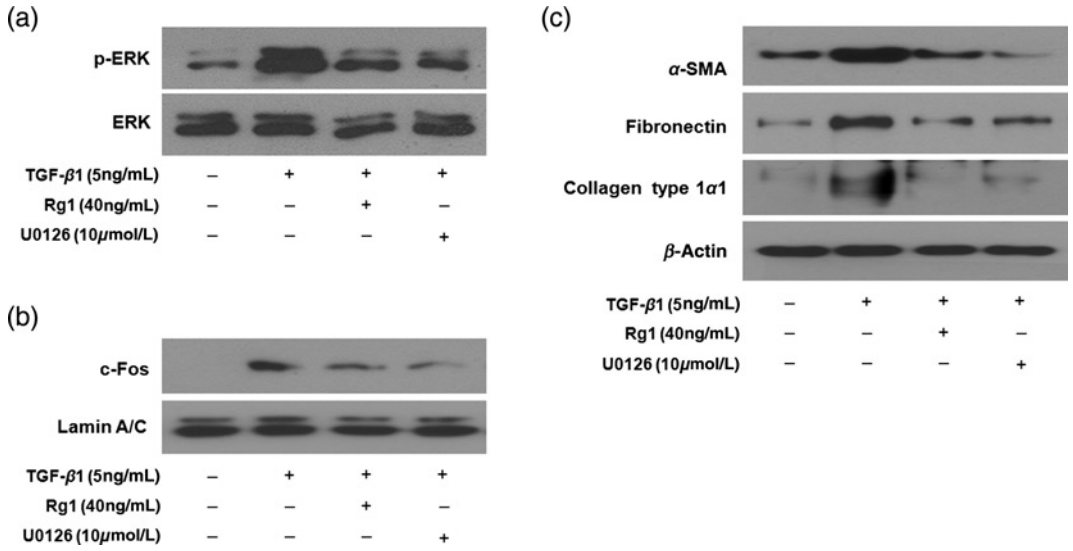

Figure 4 Effect of ERK inhibitor on myofibroblast differentiation and extracelluar matrix production in nasal polyp-derived fibroblasts (NPDFs). NPDFs induced by TGF-β1 were treated with or without Rg1 (5 or 40 ng/mL) in the absence or presence of U0126 (10 μmol/L), selective inhibitor of ERK. (a) U0126 inhibits activation of ERK induced by TGF-β1. (b) U0126 inhibits function of AP-1 induced by TGF-β1. (c) U0126 prevents TGF-β1-induced myofibroblast differentiation and extracellular matrix production. The expression of β-actin and lamin A/C are shown as internal controls. TGF-β1, transforming growth factor-β1; AP-1, activator protein 1

NPDFs. Moreover, our novel results provide direct evidence that Rg1 inhibits TGF-β1-induced myofibroblast differentiation and ECM production in NPDFs through the ERK/AP-1 pathway.

In conclusion, gensenoside Rg1 might serve as a therapeutic approach for the treatment of nasal polyp development or other diseases characterized by upregulation of TGF-β1.

Author contributions: All authors contributed to the design, interpretation of the studies, analysis of the data and review of the manuscript. J-SC conducted the experiments and wrote the manuscript. H-ML supervised the project.

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