

Epigenetic regulation of myofibroblast differentiation and extracellular matrix production in nasal polyp-derived fibroblasts

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Clinical & Experimental Allergy

Summary

Background Nasal polyposis is a multi-factorial disease associated with chronic inflammatory condition of the paranasal sinuses. Myofibroblast differentiation and extracellular matrix (ECM) accumulation are involved in the pathogenesis of nasal polyposis.

Objective The aim of this study was to study the effect of trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, on transforming growth factor (TGF)- β 1-induced myofibroblast differentiation and ECM accumulation in nasal polyp-derived fibroblasts (NPFDs).

Methods Nasal polyp-derived fibroblasts were isolated from nasal polyps of patients who have chronic rhinosinusitis with nasal polyp. TSA was treated in TGF- β 1-induced NPFDs. Expression levels of HDAC2, α -smooth muscle actin (SMA), TGF- β 1, collagen type I, acetylated Histone H3, acetylated Histone H4, phosphorylated Smad2/3 and Smad7 were determined by RT-PCR, western blot and/or immunofluorescent staining. The total collagen amount production was analysed by Sircol soluble collagen assay and contractile activity was measured by collagen gel contraction assay. HDAC2 inhibition by TSA or HDAC2 silencing was established by RT-PCR and western blot. The epigenetic effect on α -SMA gene inactivation was examined by chromatin immunoprecipitation assay. Proliferation was determined by Ki67-positive cell staining and cytotoxicity was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay.

Results The expression levels of HDAC2, α -SMA and TGF- β 1 were increased in nasal polyp tissues compared to normal inferior turbinate tissues. TSA and HDAC2 silencing inhibited expression levels α -SMA, collagen and HDAC2. TSA induced hyperacetylation of histone and suppressed opening of α -SMA gene promoter in TGF- β 1-induced NPFDs. TSA inhibited TGF- β 1-induced Smad 2/3 and rescued TGF- β 1-suppressed Smad7 signalling pathway. Finally, TSA blocked proliferation in TGF- β 1-induced NPFDs and has no cytotoxic effect in NPFDs.

Conclusions and Clinical Relevance These results suggest that HDAC inhibition is associated with myofibroblast differentiation and extracellular matrix accumulation in nasal polyposis. TSA may be useful as an inhibitor of nasal polyp growth, and thus has potential to be used as a novel treatment option for nasal polyposis.

Keywords extracellular matrix, histone deacetylase, myofibroblasts, nasal polyp-derived fibroblasts, nasal polyposis, TGF- β 1, trichostatin A

Submitted 18 April 2011; revised 05 October 2011; accepted 11 November 2011

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Introduction

Nasal polyposis is a chronic inflammatory disease of the mucous membranes in the nose and paranasal sinuses. The condition is characterized by edematous masses of inflamed mucosa prolapsing into the nose,

leading to nasal obstruction, secretion, loss of smell, headache, and reduced quality of life [1]. Although the aetiologies of nasal polyposis and the pathophysiological mechanisms leading to nasal polyp formation are still poorly understood, evidence suggests that damage to the mucosal epithelium is accompanied by

extracellular matrix (ECM) accumulation and inflammatory cell infiltration [2]. Fibroblasts, which are found in the stroma and are the cellular source of ECM proteins, are involved in the nasal polyp growth process [3]. Among the fibroblasts, myofibroblasts are an activated cell phenotype with tissue contractile properties and a high capacity for ECM protein secretion. The accumulation of ECM, which is secreted by myofibroblasts, is involved in the pathogenesis of nasal polyp formation [2].

Transforming growth factor (TGF)- β 1 plays an important role in normal airway morphogenesis and function and are involved in the pathogenesis of a variety of airway diseases [4, 5]. TGF- β 1 induces myofibroblast differentiation and ECM accumulation in nasal polyposis [3, 6]. Myofibroblasts express α -smooth muscle actin (α -SMA) and produce large amounts of ECM components [7, 8]. TGF- β 1 has been shown to be involved in the pathogenesis of chronic upper airway disorders such as nasal polyposis [1], chronic rhinosinusitis [9] and chronic hyperplastic eosinophilic sinusitis [10]. In these disorders, increased production of TGF- β 1 may lead to stromal fibrosis with fibroblast activation and differentiation.

Epigenetics, DNA methylation and chromatin modifications, are critical for generating cellular diversity, and for maintaining distinct gene expression profiles. Histone acetylation, one type of chromatin modification, is regulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC) [11, 12]. The balance of HAT and HDAC controls the levels of expression of target genes and imbalance of these enzymes can lead to several diseases [13]. Eleven HDACs that deacetylate histones are now recognized in mammalian cells and are classified into two major classes [14]. Among the 11 HDACs, HDAC2 activity was reduced in peripheral lung, bronchial biopsies and alveolar macrophages from chronic obstructive pulmonary disease patients [11, 15]. HDAC2 is also a key regulator of diabetes and TGF- β 1-induced renal injury [16]. Therefore, we focused on HDAC2 among various HDACs in nasal tissues.

According to recent findings, trichostatin A (TSA), an inhibitor of HDACs, abrogates TGF- β 1-induced ECM expression in renal [16, 17], skin [18, 19], cornea [20], liver [21] and lung fibrosis [22, 23] tissues. However, the effects of TSA inhibition on myofibroblast differentiation in nasal polyposis have not yet been reported. The purposes of this study were to investigate HDAC and α -SMA expression levels in nasal polyp tissues, to examine the inhibitory effect of TSA on TGF- β 1-induced myofibroblast differentiation and ECM accumulation, and to investigate the inhibitory mechanism of TSA in nasal polyp-derived fibroblasts (NPDFs).

Materials and methods

Reagents

Human recombinant TGF- β 1 was obtained from R&D Systems (Minneapolis, MN, USA). TSA was purchased from Sigma (St. Louis, MO, USA). TSA was dissolved in dimethylsulfoxide and then diluted to the desired concentrations with complete medium for use in the experiments.

Normal inferior turbinate and 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-2010-015). Normal inferior turbinate tissues were obtained from rhinoplasty patients and 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 and had not been treated with oral anti-allergic agents for at least 2 months.

Nasal polyp-derived fibroblasts culture

Nasal polyp-derived fibroblasts were isolated from surgical tissues by enzymatic digestion with collagenase (500 U/mL, Sigma), hyaluronidase (30 U/mL, Sigma), and DNase (10 U/mL, Sigma). Cells were cultured in DMEM containing 10% (v/v) heat-inactivated FBS (Invitrogen, Carlsbad, CA, USA), 1% (v/v) 10 000 units/mL penicillin, and 10 000 μ g/mL streptomycin (Invitrogen). The purity of obtained NPDFs was confirmed microscopically by characteristic spindle-shaped cell morphology. Approximate 95% cells in cultured NPDFs were positive for vimentin and Thy-1 (Santa Cruz Biotechnology, CA, USA), which were used for fibroblast markers, whereas negative for E-cadherin (Santa Cruz Biotechnology), epithelial cell marker. Experimental cells were obtained from the fourth cell passage.

Reverse transcription-polymerase chain reaction

Nasal polyp-derived fibroblasts (5×10^6 cells/mL) were exposed to TSA (50 nM–400 nM) with or without TGF- β 1 (5 ng/mL) for 24 hours. 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.

PCR was performed using the following primers: α -SMA (sense sequence 5'- GGTGCTGTCTCTCTATGC-CTCTGGA -3', anti-sense sequence 5'- CCCATCAGG-CAACTCGATACTCTTC -3', 322 bp), collagen type I (sense sequence 5'- CATCACCTACCACTGCAAGAAC -3', anti-sense sequence 5'- ACGTCGAAGCCGAATTCC -3', 278 bp), HDAC2 (sense sequence 5'- CATCCCATGAA-GCCTCATAGAATC -3', anti-sense sequence 5'- GCAC-CAATATCCCTCAAGTCTCC -3', 566 bp), TGF- β 1 (sense sequence 5'- GCCCTGGACACCAACTATTGCT -3', anti-sense sequence 5'- AGGCTCCAAATGTAGGGGCAGG -3', 160 bp), and GAPDH (sense sequence 5'- GTGGA-TATTGTTGCCATCAATGACC -3', anti-sense sequence 5'- GCCCAGCCTTCTTCATGGTGGT -3', 271 bp). The gels were captured and visualized using Molecular Imager ChemiDoc XRS+ (Bio-Rad, Hercules, CA).

Western blot analysis

Nasal polyp-derived fibroblasts were exposed to TSA with or without TGF- β 1. NPDFs lysates were subjected to SDS-PAGE and transferred onto PVDF membranes (Millipore Inc., Billerica, MA, USA). Membranes were blocked with 5% skim milk solution and were incubated with the following antibodies: α -SMA (Chemicon, Millipore Inc.), acetylated histone H4, acetylated histone H3 (Upstate, Millipore Inc.), TGF- β 1 (Cell Signaling Technology Inc., Boston, MA, USA), HDAC2, Collagen type I, Histone H3, Histone H4, pSmad2/3, Smad7, β -actin or laminA/C (Santa Cruz Biotechnology). Blots visualized with HRP-conjugated secondary antibodies and ECL system (Pierce, Rockford, IL, USA).

Immunofluorescent staining

Five- μ m-thick sectioned tissues or NPDFs were fixed with 4% paraformaldehyde. The tissues and NPDFs were permeabilized with 0.2% TritonX-100 in 1% bovine serum albumin (BSA) for 10 min, blocked with 5% BSA for one hour at room temperature and incubated overnight at 4°C with anti- α -SMA antibody (Chemicon), anti-HDAC2 (Santa Cruz Biotechnology), anti-TGF- β 1 antibody (Cell Signaling Technology Inc.) and/or pSmad2/3, Smad7 (Santa Cruz Biotechnology). The tissues and NPDFs were then incubated with goat anti-mouse Alexa 488 (Invitrogen) or goat anti-rabbit Alexa 555 (Invitrogen) secondary antibodies or rhodamine-phalloidin (Invitrogen). Finally, coverslips were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Each stained tissues were captured and visualized using a confocal z-stack laser scanning microscopy (LSM700, Zeiss, Oberkochen, Germany) and the NPDFs were taken fluorescence microscopy (IX71, Olympus, Tokyo, Japan) or confocal microscopy.

Sircol soluble collagen assay

The Sircol Collagen Assay (Biocolor Ltd, Belfast, UK) is a quantitative dye binding method designed for the analysis of acid soluble collagens. The assay was performed according to the manufacturer's recommendations.

Collagen gel contraction assay

Rat-tail tendon collagen type I (RTTC) was purchased from BD Bioscience (Bedford, MA, USA). NPDFs were detached by 0.05% trypsin and suspended in serum-free DMEM. Collagen gels were prepared as previously described by mixing RTTC, serum-free DMEM, and NPDFs. NPDFs were then mixed with the neutralized collagen solution (pH 7.4) so that the final cell density in the collagen solution was 3×10^5 cells/mL, and the final concentration of collagen was 0.75 mg/mL. Aliquots (0.5 mL/well) of the mixture of cells in collagen were cast into each well of 24-well tissue culture plates and allowed to polymerize at room temperature, generally completed in 20 min. After polymerization, the gels were gently released from the 24-well tissue culture plates and transferred into 6-well culture plate, which contained 1.5 mL serum free-DMEM with TGF- β 1 and/or TSA. The gels were then incubated at 37°C in a 5% CO₂ atmosphere for 3 days. The area of each gel was measured with an Image J analyser (NIH, Bethesda, MA, USA). Data are expressed as the percentage of area compared with the initial gel area.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation assay was performed using an EZ ChIP kit (Upstate, Millipore Inc.) according to the manufacturer's instructions. Cross-linked DNA-protein complexes were immunoprecipitated using anti-acetylated histone H4 antibody (Upstate). Free DNA was purified by semi-quantitative PCR using the following primers: -136/-58 α -SMA promoter sequence, 5'- AGTTTGTG CTGAGGTCCCTATATG -3' and 5'- TTCCCAAACAAGGAGCAAAGA -3'. Normal rabbit IgG was used as a negative control. Average values of eluates were normalized to average values of inputs.

RNA interference

Nasal polyp-derived fibroblasts were plated in 6-well plates and grown to 60% to 70% confluence. siRNA of control or HDAC2 were synthesized by Santa Cruz Biotechnology. NPDFs were transiently transfected with siRNA using LipofectamineTM RNAiMax (Invitrogen) according to the manufacturer's instructions. These siRNA transfected cells were treated with TGF- β 1, then

these NPDFs were used for RT-PCR and western blot analysis.

Immunostaining procedure for proliferation measurement

Nasal polyp-derived fibroblasts were seeded in coverslip and cultured. After fixation with 4% paraformaldehyde at room temperature for 30 min, the NPDFs on coverslips were applied with the anti-Ki67 monoclonal antibody (Santa Cruz Biotechnology). Then the NPDFs were incubated with goat anti-mouse Alexa 555 secondary antibody (Invitrogen). Finally, coverslips were counterstained with DAPI. The coverslips were mounted and observed with confocal laser scanning microscopy. Ki67-positive cells and DAPI-stained cells were counted and analysed with four high-power fields ($\times 100$).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

The cytotoxic effect of TSA was evaluated by the MTT assay. TSA was incubated at various concentrations (0–800 nM) for 48 hours. After this period, the cells were treated with MTT solution (Sigma) for 5 hours, and DMSO was added to each well. The plates were agitated on a plate shaker and the optical density then read using by a fluorescence microplate reader (F2000; Hitachi, Ltd., Tokyo, Japan).

Statistical analysis

The results are obtained from at least three independent experiments. The statistical significance of the differences between control and experimental data was analysed using the Student t-test or one-way ANOVA followed by Tukey's test (GraphPad Prism, version 5, GraphPad Software, San Diego, CA). Significance was established at the 95% confidence level. *P*-values < 0.05 were accepted as statistically significant.

Results

Histone deacetylase2, α -smooth muscle actin and transforming growth factor- β 1 expression levels are increased in nasal polyp tissues

α -SMA is a well known marker of myofibroblast differentiation. RT-PCR analysis showed that the mRNA expression levels of HDAC2, α -SMA and TGF- β 1 were increased in nasal polyp tissues compared to normal inferior turbinate tissues (Figs 1a and b). Western blot analysis showed that protein expression levels of HDAC2, α -SMA and TGF- β 1 were higher in nasal polyp tissues than in normal inferior turbinate tissues (Figs 1c

and d). α -SMA was expressed at endothelial cells in normal inferior turbinate tissues and was significantly expressed at fibroblasts as well as endothelial cells in nasal polyp tissues. Expression of TGF- β 1 was higher in nasal polyp tissues than normal inferior turbinate tissues and was localized and concentrated in inflammatory cells (Fig. 1d). The results demonstrate that both transcript and protein expression levels of HDAC2, α -SMA and TGF- β 1 increased in nasal polyp tissues.

Trichostatin A influences morphology of transforming growth factor- β 1-induced nasal polyp-derived fibroblasts

TGF- β 1 is a potent stimulus for the differentiation of fibroblasts into myofibroblasts in various organs such as lung, skin and kidney. TSA is an inhibitor of histone deacetylase and prevents myofibroblast differentiation. In a previous study, TGF- β 1-induced lung myofibroblast has larger and polygonal morphological changes compared to control [24]. To identify whether TGF- β 1 induces morphological phenotype changes in NPDFs and TSA inhibits the TGF- β 1 effect, NPDF cells were treated with TGF- β 1 with or without TSA and then stained with rhodamine-phalloidin and α -SMA (Fig. 2). Rhodamine-phalloidin is used for cell cytoskeleton staining. Control NPDFs appeared as spindle-shaped and elongated cells in shape. However, TGF- β 1-induced NPDFs were observed as spread-out, flattened and dispersed cells in shape. TSA treatment in TGF- β 1-induced NPDFs returned cells to the bipolar and elongated shape, as observed in control NPDFs. Taken together, these results suggest that TSA treatment reverses morphological changes induced by TGF- β 1.

Trichostatin A inhibits transforming growth factor- β 1-induced myofibroblast differentiation and extracellular matrix production in nasal polyp-derived fibroblasts

To investigate the effect of TGF- β 1 and TSA on myofibroblast differentiation and ECM production, NPDFs were treated with TGF- β 1 with or without TSA and were determined for α -SMA and collagen expression. Expression levels of α -SMA mRNA (Figs 3a and b) by RT-PCR and protein (Figs 3c and d) by western blot and immunofluorescent staining were increased in TGF- β 1-treated NPDFs compared to the control. In addition, mRNA expression levels of collagen type I was determined by RT-PCR. Collagen protein production was measured by sircol soluble collagen assay. Contractile activity of myofibroblasts was examined by collagen gel contraction assay. Expression levels of collagen mRNA and protein increased in TGF- β 1-treated NPDFs (Figs 3e, f and g). TSA significantly reduced TGF- β 1-induced α -SMA

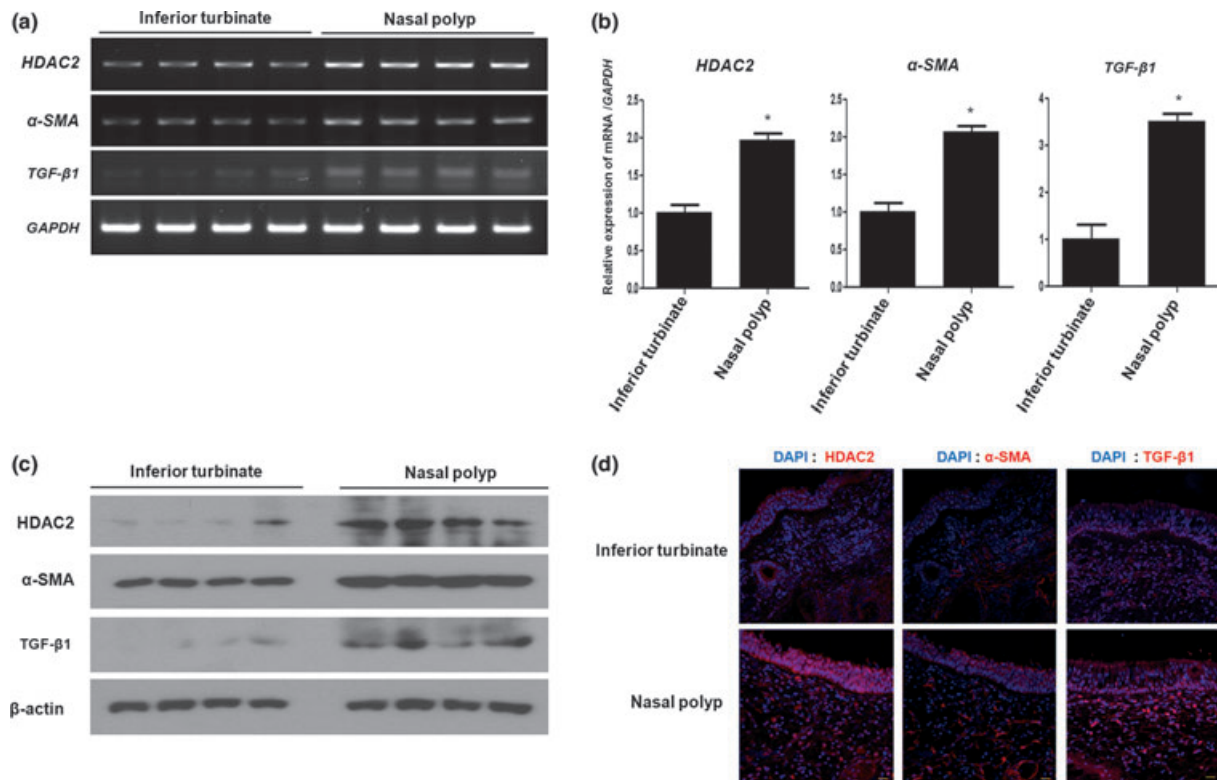


Fig. 1. Expression of histone deacetylase (HDAC)2, α -smooth muscle actin (α -SMA) and transforming growth factor (TGF)- β 1 in normal inferior turbinate and nasal polyp tissues. (a) The expression levels of HDAC2, α -SMA and TGF- β 1 mRNA by RT-PCR. (b) Quantification of mRNA expression levels. Values are means $\pm$ SEM of independent samples. Asterisks (*) indicate statistically significant difference ($P < 0.01$). (c) The expression levels of HDAC2, α -SMA and TGF- β 1 proteins by western blot. (d) The expression patterns and localization of HDAC2, α -SMA and TGF- β 1 proteins by immunohistochemical staining. Images were captured and visualized by confocal z-stack laser scanning microscope. Scale bar, 40 μ m.

and collagen expression at both the RNA transcript and protein levels (Fig. 3). These results indicate that TSA inhibits TGF- β 1-induced myofibroblast differentiation and ECM production in NPFDs.

Trichostatin A inhibits histone deacetylase2 expression and is involved in acetylation of histones

TSA is one of the HDAC inhibitors and induces hyperacetylation of histones. To investigate inhibition of HDAC and induction of hyperacetylation by TSA, we demonstrated the expression levels of HDAC2 by RT-PCR and western blot analysis. TGF- β 1 induced HDAC2 expression levels in both mRNA and protein, however, TSA inhibited the effect of TGF- β 1 (Figs 4a and b). Acetylated histone H3 and H4 expression were examined by western blot analysis. TSA induced the hyperacetylation of histone H3 and H4 in a dose-dependent manner (Fig. 4c). These results indicate that TSA significantly suppresses HDAC and induces hyperacetylation of histone. To determine whether TSA-induced hyperacetylation of histones was associated with alteration of transcriptional activity, a chromatin immunoprecipitation assay was performed. This assay is

used to examine promoter opening of an α -SMA gene involved with acetylated histone H4. The chromatin immunoprecipitation assay showed that the α -SMA promoter was opened in TGF- β 1-treated NPFDs and TSA significantly reduced TGF- β 1-induced α -SMA promoter opening (Fig. 4d). These data imply that TGF- β 1 induces activation of the α -SMA gene transcription by opening the chromatin, and inhibition of HDAC and hyperacetylation by TSA is associated with the down-regulation of the α -SMA gene transcription.

Inhibition of histone deacetylase2 suppresses transforming growth factor- β 1-induced myofibroblast differentiation and extracellular matrix production in nasal polyp-derived fibroblasts

To determine effect of silencing of HDAC2 by siRNA on TGF- β 1-induced myofibroblast differentiation and ECM production, NPFDs were transfected with siControl or siHDAC2 and were determined expression level of HDAC2, α -SMA and collagen type I. Expression levels of HDAC2 mRNA and protein were decreased in siHDAC2-transfected NPFDs compared to the control (Figs 5a and b). We treated with TGF- β 1 after

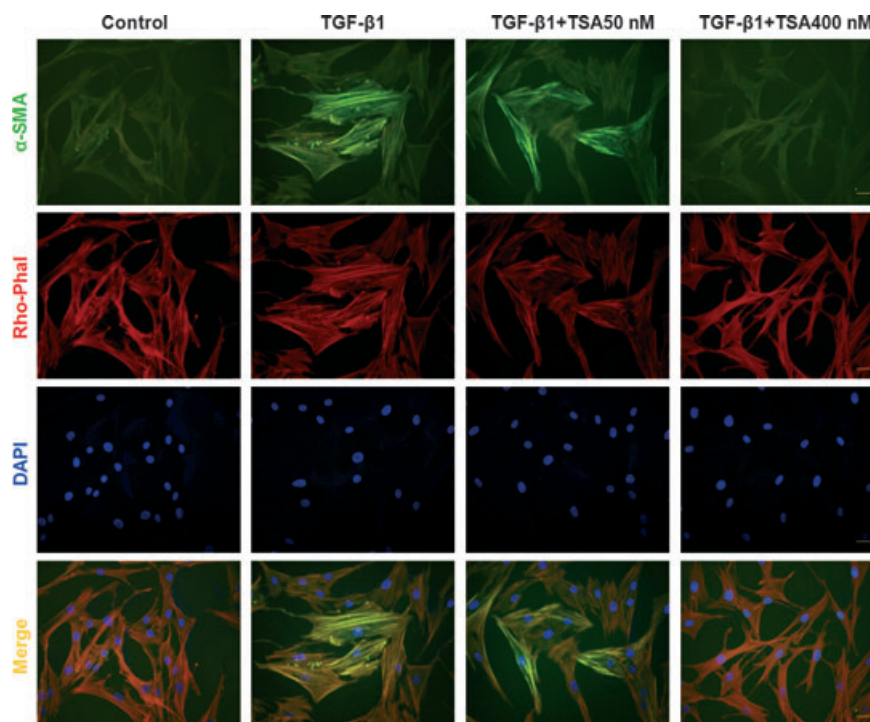


Fig. 2. The morphological changes by trichostatin A (TSA) in transforming growth factor (TGF)- β 1- induced fibroblasts. The expression of α -smooth muscle actin (α -SMA) (green) protein was assessed by immunofluorescent staining. Rhodamine-phalloidin (red) staining was employed for verification of cell morphology. The nucleus was counterstained using 4',6-diamidino-2-phenylindole (DAPI) (blue). Scale bar, 50 μ m.

transfection of siControl or siHDAC2 and measured α -SMA and collagen expression. After TGF- β 1 treatment, mRNA and protein expression levels of α -SMA, collagen type I and HDAC2 were increased in siControl-transfected NPFDs. However, although siHDAC2-transfected NPFDs were treated with TGF- β 1, the expression levels of α -SMA, collagen type I and HDAC2 were not increased in both mRNA and protein (Figs 5c and d). These data suggest that HDAC2 is important for TGF- β 1-induced myofibroblast differentiation and ECM production.

The abrogation of myofibroblast differentiation by trichostatin A is related to phosphorylation of Smad2/3

Phosphorylation of Smad 2/3 is an important step in the signalling pathway of TGF- β 1- induced gene transcription. Phosphorylated Smad2/3 are activated and translocated from the cytoplasm into the nucleus. To investigate the mechanism by which TSA inhibits myofibroblast differentiation, the effect of phosphorylated Smad2/3, which is two major TGF- β 1-activated signalling proteins, was identified using western blots. TGF- β 1 activated phosphorylation of Smad2/3, but TSA inhibited the TGF- β 1- induced phosphorylation of Smad2/3 in a dose-dependent manner (Fig. 6a). To identify the translocation of phosphorylated Smad2/3, cells were stained with pSmad2/3 antibody and observed under confocal

microscopy. Significantly increased translocation of pSmad2/3 from the cytoplasm to nucleus was observed in TGF- β 1-treated NPFDs, but translocation of pSmad2/3 was blocked in TSA-treated NPFDs (Fig. 6c). Smad7 block the phosphorylation of Smad2 or Smad3, thus inhibiting TGF- β signalling in lung [7]. To determine TSA induce expression of Smad7, NPFDs were treated with TGF- β 1 and/or with TSA and measured by western blot analysis and immunofluorescent staining. Expression of Smad7 was decreased in TGF- β treated NPFDs, but the expression was rescued in TSA-treated NPFDs (Figs 6b and c). These data recommend that TSA inhibits TGF- β 1-induced activation and translocation of pSmad2/3, and induces expression of Smad7.

Trichostatin A blocks proliferation and has no cytotoxic effect in nasal polyp-derived fibroblasts

TGF- β 1 induces proliferation in human renal fibroblasts [25]. To provide whether TGF- β 1 induces proliferation of NPFDs, NPFDs were treated with TGF- β 1 and stained with Ki67 and we counted Ki67-positive cells. Ki67-positive cells were increased by TGF- β 1 treatment, however, TSA dramatically inhibited the effect of TGF- β 1-induced proliferation (Fig. 7a). To examine the possible cytotoxicity of TSA in NPFDs cultures, we performed MTT assay. NPFDs were treated with TSA upto 800 nM and cell survival was measured by the MTT

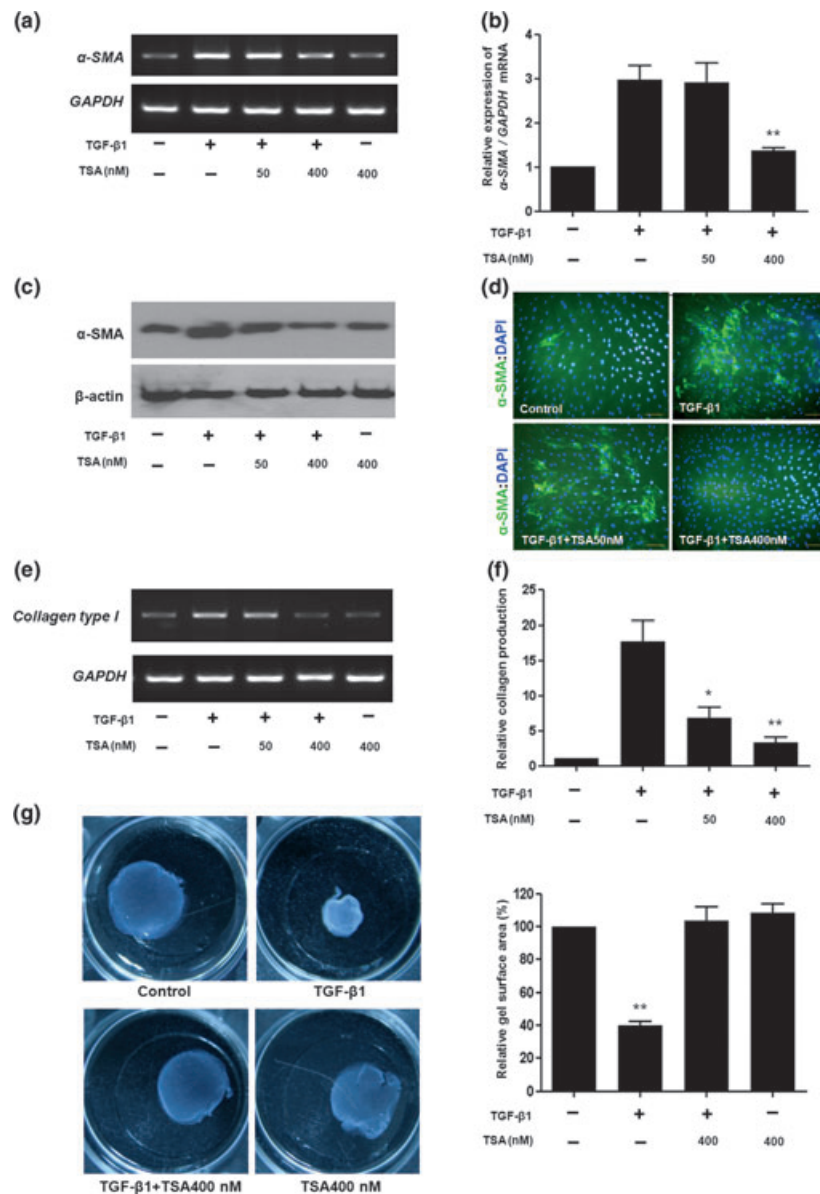


Fig. 3. Effect of trichostatin A (TSA) on myofibroblast differentiation and extracellular matrix (ECM) production in transforming growth factor (TGF)- β 1-induced nasal fibroblasts. (a) Semi-quantitative RT-PCR of α -smooth muscle actin (α -SMA) mRNA transcript (b) quantitative analysis of α -SMA mRNA transcript (c) western blot of α -SMA protein (d) immunofluorescent staining of α -SMA protein expression was determined. (e) Collagen type I mRNA transcript was examined by semi-quantitative RT-PCR and (f) total collagen was measured with Sircol assay kit. (g) Contractile activity was assessed by collagen gel contraction assay and the contraction area was measured. Asterisks (*, $P < 0.05$; **, $P < 0.01$) indicate statistically significant difference. The results are obtained from at least three independent experiments. Scale bar, 100 μ m.

assay (Fig. 7b). These data indicate that TGF- β 1-induced proliferation is blocked by TSA and 400 nM of TSA has no cytotoxic effect.

Discussion

This study demonstrates the role of epigenetic changes by histone modifications in myofibroblast differentiation and ECM production, and the inhibitory effects of TSA on nasal polyposis development in NPDFS.

Epigenetics, DNA methylation and chromatin modifications are critical for maintaining distinct gene expression profiles [13]. Noh et al. showed that HDAC2 was increased in a diabetic rat model and TGF- β 1-induced NRK52-E cells and HDAC2 plays an important role in the development of ECM accumulation [16]. We have shown that expression of HDAC2 increases in nasal polyp tissues compared to normal nasal inferior turbinates. To our knowledge, this is the first study to show that HDAC2 expression level is increased in nasal polyp tissues.

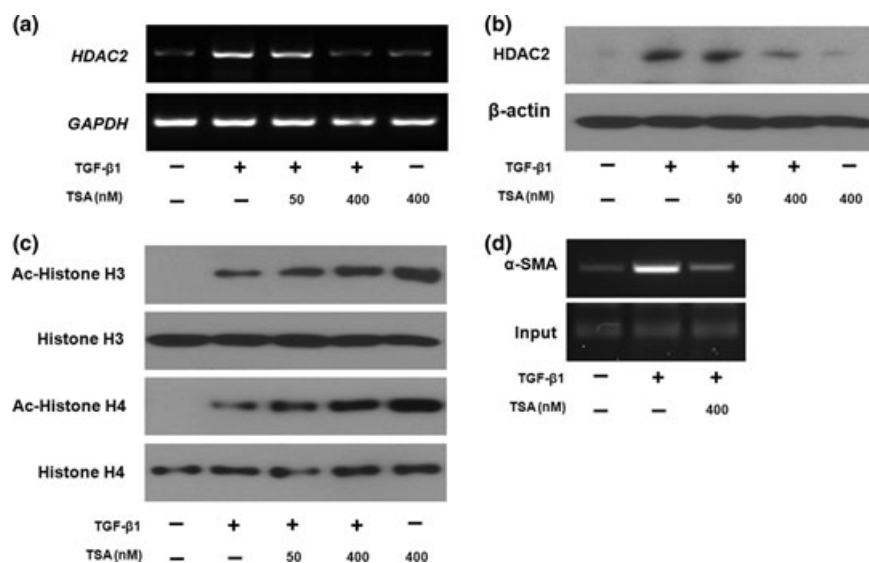


Fig. 4. Effect of trichostatin A (TSA) on histone deacetylase (HDAC)2 inhibition and hyperacetylation of histones in nasal fibroblasts. (a) Semi-quantitative RT-PCR of HDAC2 mRNA transcript and (b) western blot of HDAC2 protein were determined. (c) Hyperacetylation of histone H3 and H4 by TSA was measured by western blot analysis using acetylated histone 3 and 4 (Ac-Histone 3 and Ac-Histone 4) antibody. Histone 3 and Histone 4 were used for internal control. (d) Chromatin immunoprecipitation assay was performed using a specific antibody against acetyl histone H4 (Ac-Histone 4) and a specific primer for the α -smooth muscle actin (α -SMA) promoter region. Input data in the chromatin immunoprecipitation assay was used for internal control. TGF, transforming growth factor.

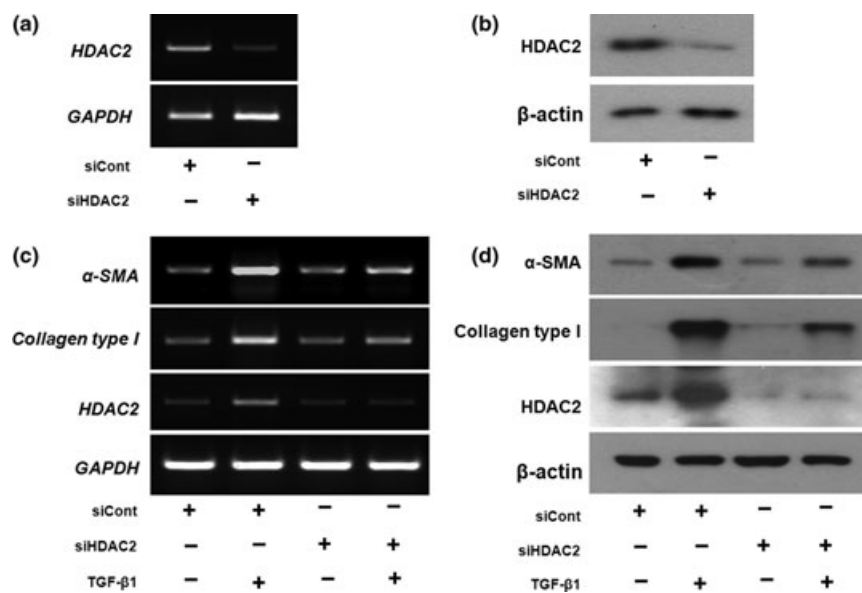


Fig. 5. Effect of histone deacetylase (HDAC)2 silencing on myofibroblast differentiation and extracellular matrix production in transforming growth factor (TGF)- β 1- induced nasal fibroblasts. After siControl or siHDAC2 transfection, HDAC2 silencing was determined in (a) mRNA transcript level by RT-PCR and (b) protein level by western blot. Expression of α -SMA, collagen type I and HDAC2 were assessed by (c) RT-PCR and (d) western blot in TGF- β 1- induced nasal fibroblasts.

Myofibroblast differentiation and ECM accumulation are involved in nasal polyp formation [1, 2]. TGF- β 1-induced myofibroblast differentiation produces large amounts of α -SMA and ECM production [3–5, 7–9]. We demonstrated that both mRNA and protein expressions of α -SMA and TGF- β 1 were increased in nasal polyp

tissues compared to normal nasal inferior turbinate tissues.

We showed that expression levels of HDAC2, α -SMA and TGF- β 1 are increased in nasal polyp tissues, which suggested that HDAC2 regulates myofibroblast differentiation and plays a role in nasal polyp formation.

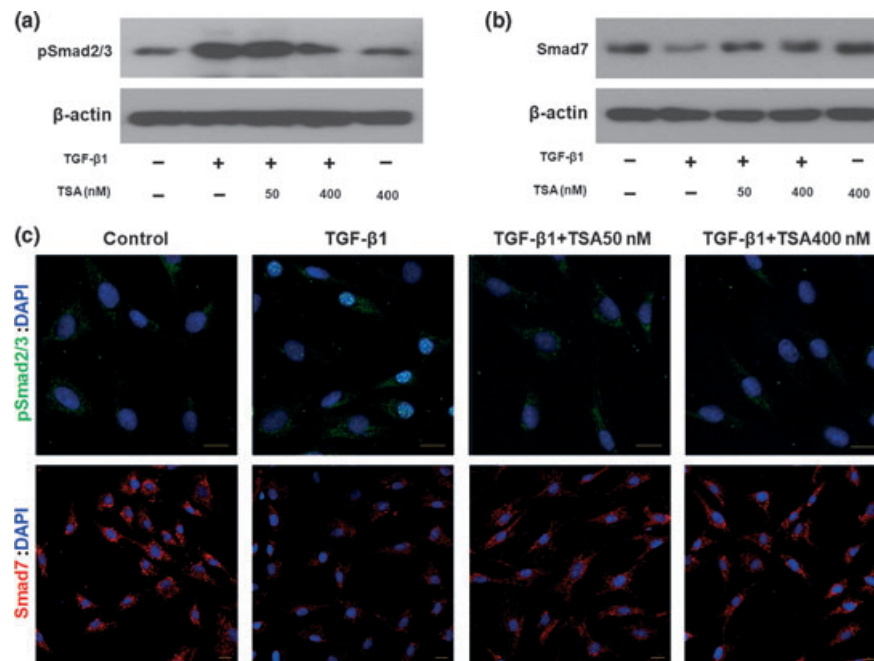


Fig. 6. The inhibitory effect of trichostatin A (TSA) on phosphorylated Smad2/3 in transforming growth factor-(TGF)β1-induced fibroblasts. (a) Expression of phosphorylated Smad2/3 was determined by western blot. (b) Protein expression of Smad7 was measured by western blot analysis. (c) Nuclear translocation of phosphorylated Smad2/3 protein and expression of Smad7 were confirmed by immunofluorescent staining. Images were captured and visualized by confocal laser scanning microscope. Scale bar, 20 μm.

Several recent studies have suggested that TSA, an HDAC inhibitor, suppresses myofibroblast differentiation in hepatic stellate cells [21], skin fibroblasts [18, 19], kidney cells [16, 17], lung fibroblasts [22, 23] and corneal fibroblasts [20]. In this study, we demonstrated that TSA inhibits TGF-β1-induced morphological changes, myofibroblast differentiation and ECM accumulation in NPFDs.

In our study, TSA inhibited expression of HDAC2 and increased the hyperacetylation caused by the inhibition of HDAC function. Although hyperacetylation by TSA is generally associated with transcriptional activation, it is not certain how hyperacetylation by TSA can lead to transcriptional repression of profibrotic genes. We demonstrated that α-SMA promoter was opened in TGF-β1-treated NPFDs and TSA significantly inhibited TGF-β1-induced α-SMA promoter opening. These data suggest that TGF-β1 induces α-SMA gene transcription by opening the chromatin and TSA is associated with the down-regulation of the α-SMA gene transcription. In addition, the results from the present study show that changes in cellular histone acetylation activities modulate α-SMA promoter opening in response to TGF-β1 and TSA.

The phosphorylation of Smad2/3 is an important signalling step in TGF-β1 induced gene transcription. Phosphorylated Smad2/3 are activated and translocated into the nucleus from the cytoplasm [5, 26] where they activate α-SMA gene expression [27]. Smad7, inhibitory

molecules of TGF-β signalling, block the phosphorylation of Smad2 or Smad3 [7]. In mice with bleomycin-induced pulmonary fibrosis, expression of the inhibitory Smad7 decreases lung fibrosis [28]. TSA decreased the levels of the mRNAs of Smad7 in retinoblastoma cell lines [29]. In the present study, we have showed that TSA inhibited activation and translocation of Smad2/3 activated by TGF-β1 and increased expression of Smad7. Therefore, our results suggest that TSA influences TGF-β1 to signal its downstream partners such as Smad molecules. Recent studies have shown that corepressors such as Ski, SnoN, TGIF and TGIF2 are histone deacetylase-associated proteins that interact directly with Smad2 and/or Smad3 [30, 31]. These corepressors may be involved in repression of TGF-β1-induced fibrotic gene transcription, and TSA may inhibit the effect of TGF-β1 by interacting with these corepressors.

Nasal polyp tissues have increased mRNA levels of TGF-β1 than normal nasal mucosa [32]. We compared to normal nasal fibroblasts and NPFDs. The morphology of normal fibroblasts was similar to NPFDs. The myofibroblast differentiation and ECM production and inhibitory effects by TSA in normal nasal fibroblasts had effect like NPFDs (data not shown). These results indicate that TSA has similar inhibitory effect on myofibroblast differentiation and ECM production in both normal nasal fibroblasts and NPFD.

By inhibiting the deposition of ECM in nasal polyposis, TSA may potentially be useful as an agent for

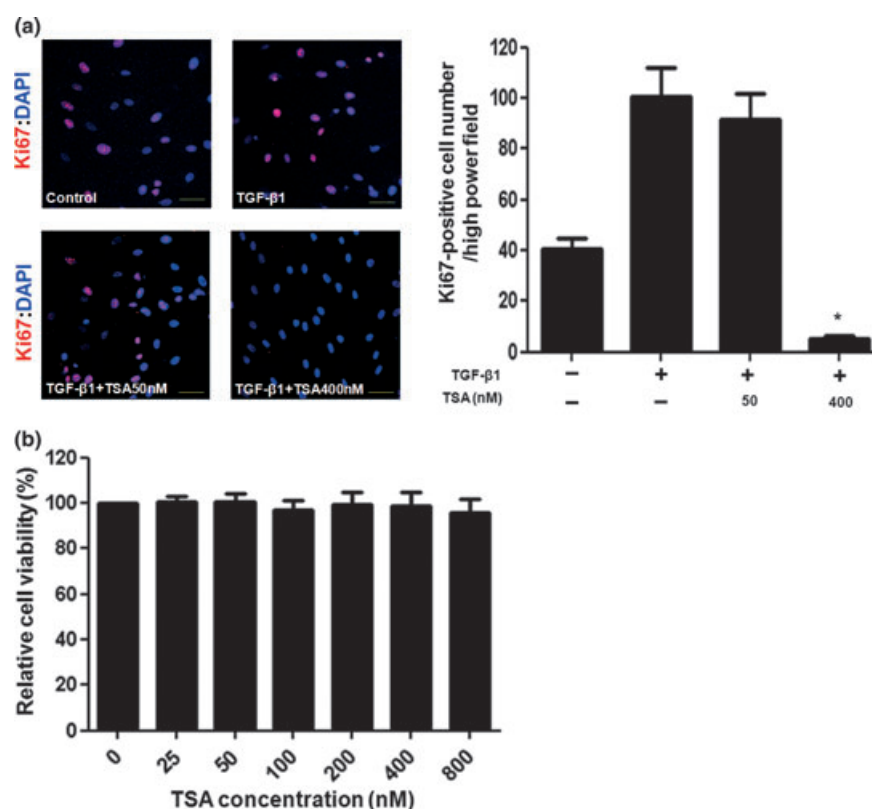


Fig. 7. Effect of trichostatin A (TSA) on proliferation in transforming growth factor (TGF)- β 1-induced nasal fibroblasts and on cytotoxicity. (a) nasal polyp-derived fibroblasts (NPDFs) were treated with TGF- β 1 with or without TSA and stained with Ki67 antibody. Ki67-positive cells were examined by immunofluorescent staining and counted. Scale bar, 50 μ m. (b) NPDFs were treated with TSA upto 800 nM and cytotoxicity of cells was measured by the MTT assay.

inhibiting the growth of nasal polyposis. However, further studies into the inhibitory mechanisms of TSA and the effects of TSA for the treatment of nasal polyposis *in vivo* are needed.

In conclusion, we demonstrated that HDAC2 and α -SMA are increased in nasal polyposis and that inhibition of HDAC2 by TSA reduces TGF- β 1-induced myofibroblast differentiation and ECM accumulation in NPDFs. We also demonstrated that histone acetylation is associated with myofibroblast differentiation and that histone deacetylase inhibitors such as TSA may be clinically useful for inhibition of nasal polyp development.

Acknowledgements

This study was supported by a grant from the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A090084) and supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), which is funded by the Ministry of Education, Science, and Technology (2010-0010279).

Conflict of interest None of the authors report a conflict of interest.

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