

Notoginsenoside R1 prevents EMT of BEAS-2B cells via suppressing the TGF- β 1/Smad pathway

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Research Article

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Abstract

Notoginsenoside R1 (NR1) is a main effective component of *Panax notoginseng* (Burk) F. H. Chen, which can inhibit lung fibroblast cell inflammatory injury. In the present study, a chronic obstructive pulmonary disease (COPD) model of BEAS-2B cells treated with cigarette smoke extract (CSE) was established *in vitro* to investigate the effects of NR1 on epithelial-mesenchymal transition (EMT). The results revealed that the expression levels of α -smooth muscle actin (α -SMA, a mesenchymal marker) were increased, and the levels of E-cadherin (an epithelial marker) were decreased due to exposure to CSE, which was significantly reversed by NR1. Moreover, the components of the TGF- β 1/Smad pathway, including TGF- β 1 and phosphorylation of Smad2 and Smad3 induced by CSE, were also downregulated through NR1. In addition, NR1 inhibited the binding activity of phosphorylated (p)-Smad2 and p-Smad3 complexes to plasminogen activator inhibitor 1. In conclusion, NR1 relieves EMT caused by CSE in BEAS-2B cells via suppressing the TGF- β 1/Smad pathway.

Introduction

Chronic obstructive pulmonary disease (COPD), which is characterized by constant irreversible airflow limitation, is a leading cause of disease and death worldwide[1]. COPD is a preventable condition associated with airway inflammation and remodeling, airway damage and emphysema. It is likely to be caused by cigarette smoke exposure and other toxins[2, 3]. This is in contrast to the perspective of airway remodeling in asthma, while airway obstruction is mainly caused by small airway remodeling, which further causes airflow limitation[4].

According to recent studies, epithelial-mesenchymal transition (EMT) is a process where epithelial cells undergo cytoskeleton remodeling, the connection between cells is lost, and a mesenchymal phenotype with excessive extracellular matrix deposition is acquired. It participates in the respiratory structural remodeling in COPD[5]. The transition of epithelial cells results in enhanced mobility, and cells cross the basal membrane, causing the release of extracellular matrix proteins to facilitate fibrosis and enhanced tissue remodeling. TGF- β 1/Smad is a key player in inducing EMT[6–8]. A previous study showed that TGF- β 1 expression was often increased in the airway wall of patients with COPD, and the phosphorylation of Smad2/3 proteins was associated with smoking, especially smokers with COPD[9]. Therefore, investigating the mechanism of pathways associated with TGF- β 1 in COPD can offer promising treatment targets to suppress airway remodeling.

The roots of *Panax notoginseng* (*P. notoginseng*) (Burk) F.H. Chen is a herbal medicine widely used in Asian countries in managing pulmonary function and cardiovascular disease[10]. *P. notoginseng* saponins (PNS), the main active ingredient of Panax, can suppress platelet aggregation, enhance cardiac angiogenesis and facilitate left ventricular diastolic function, as well as resist inflammation and suppress vascular intimal hyperplasia and vascular smooth muscle cell proliferation[11–14]. Notoginsenoside R1 (NR1) is a novel bisdesmosidic 20 (S)-protopanaxatriol saponin, which is considered as a distinct saponin in *P. notoginseng*. However, it does not exist in other ginseng species[15]. Increasing evidence

has shown that NR1 exerted cardioprotective, neuroprotective, anti-apoptotic, anti-inflammatory, and anti-oxidative effects[16, 17]. However, the effects of NR1 on airway remodeling in COPD remain to be elucidated. In the present study, the effects of NR1 on an EMT model caused by cigarette smoke extract (CSE) *in vitro* and its possible mechanism were assessed from the perspective of the TGF- β 1/Smad pathway.

Materials And Methods

Cell culture

BEAS-2B cells were purchased from the American Type Culture Collection. Cells were cultured in a complete BEAS-2B culture medium (BEBM) and incubated at 37°C, 5% CO₂. The complete BEBM (Lonza Group Ltd.) is prepared from supplementary BEBM basic medium and BEGM SingleQuotes kit (Lonza Group Ltd.). BEAS-2B of 3 passages with 80% confluence was used in the experiments.

Exposure To Cigarette Smoke

A peristaltic pump was used to burn the unfiltered cigarette. From the beginning of ignition until the end of a cigarette burn, cigarette smoke slowly bubbled into 10 ml of complete BEBM, which lasted about 6 min. The resulting medium was adjusted to pH 7.4, bypassing 0.22- μ M millex filters (EMD Millipore) for sterile filtration and defined as 100% CSE. BEAS-2B cells were treated with 0, 3.125, 6.25, 12.5, 25, 50, 100 μ g/ml of NR1 (Shanghai Tauto biotech company, Shanghai, China) pretreatment for 24 h. Then, BEAS-2B cells were exposed to 2.5% CSE (treatment group) or an equal volume of purified air (control group) at 37 °C and 5% CO₂ for 4 h.

Wound Healing Assay

BEAS-2B cells migration was evaluated by wound healing assay. Briefly, NR1 and 2.5% CSE treated BEAS-2B cells were seeded into 6-well plates at a density of 5×10^5 cells/well. Once cells reached 80% confluence, a scratch was made using a 200 μ l pipette tip, and a serum-free medium was used. After 0, 12, 24 and 48 h of culture, BEAS-2B cells were observed under an inverted microscope (Olympus Corporation), and the distance between wounds was recorded.

Reverse Transcription-quantitative Pcr (Rt-qpcr)

TRIzol® reagent was used to extract total RNA in BEAS-2B cells according to the manufacturer's instructions. RT-qPCR amplification was conducted to analyze RNA expression of TGF- β 1, E-cadherin, N-cadherin, α -smooth muscle actin (SMA) and β -actin using SYBR Green reagent (Takara Biotechnology Co., Ltd.) on the Stratagene 3005p system (Agilent Technologies, Inc.). The housekeeping genes, including β -actin, were used as the internal reference. The relative expression of genes was calculated

using the $2^{-\Delta\Delta C_q}$ method. The following primer pairs were used for the qPCR: E-cadherin (NM_001317185.2) forward, 5'-ATGAGTGTCCCCCGGTATCTT-3' and reverse, 5'-TCAGCCGCTTTCAGATTTTCA-3'; N-cadherin (XM_017025514.2) forward, 5'-AGCCAACCTTAAGTGAAGAGT-3' and reverse, 5'-GGCAAGTTGATTGGAGGGATG'; TGF- β 1 (NM_000660.7) forward, 5'-GGCCAGATCCTGTCCAAGC-3' and reverse, 5'-GTGGGTTTCCACCATTAGCAC-3'; α -SMA (NM_001613.4) forward, 5'-GACAGCTACGTGGGTGACGAA-3' and reverse, 5'-TTTTCCATGTCTGTCCTCCAGTTG-3' and β -actin (NM_001101.5) forward, 5'-GTGGGGCGCCCCAGGCACCAGGGC-3' and reverse, 5'-CTCCTTAATGTCACGCACGATTTC-3'.

Immunofluorescence Assay

Cells were mounted in ice-cold methanol and permeabilized with PBS supplemented with 0.25% TritonX-100 at room temperature for 10 min. Non-specific binding sites were blocked with 1% BSA (Sigma-Aldrich; Merck KGaA) in PBS with 0.05% Tween-20 for 1 h. The cells were incubated with E-cadherin, N-cadherin and α -SMA antibodies and incubated again with FITC-or tetramethylrhodamine-conjugated secondary antibodies. Following washing, nuclei were stained with DAPI (Invitrogen; Thermo Fisher Scientific, Inc.). Finally, the results were observed under an SP5 Leica confocal microscope using the Leica Application Suite software (v14.0.0162; Leica Microsystems GmbH). Isotopes were used as the control instead of antibodies.

Western Blot Assay

Western blot assay

Cells were homogenized with liquid nitrogen and resuspended in RIPA buffer(P0013B, beyotime biotechnology) and an optional protease inhibitor cocktail (Roche Applied Science). Protein concentration was then detected using NanoDrop (Epoch, BioTek). Following protein separation on 8–10% SDS-PAGE for 90 min at 100 V, the protein samples were transferred onto a PVDF membrane for 1 h through a wet transfer at 250 mA. Subsequently, the PVDF membrane was incubated with E-cadherin, N-cadherin, α -SMA, Smad2/3, p-Smad2/3, Smad7 and β -actin antibodies, followed by incubation with HRP-conjugated secondary antibodies. Protein signals were developed using an ECL kit. The optical density was evaluated based on the loading control, and the ratio was obtained via Quantity One software(version 4.3.0 The Discovery Series).

Dual Luciferase Reporter Assay

Following mutation of the putative binding site in the promoter region of plasminogen activator inhibitor 1 (PAI-1) using a mutagenesis kit, the vector was inserted with wild-type (WT) and mutant (Mut) sequences for the construction of luciferase reporter plasmids according to the manufacturer's instructions (Promega Corporation). The dual luciferase reporter kit (Promega Corporation) was used to

measure luciferase activity. The promoter region of PAI-1 cDNA with one putative target site for phosphorylated (p)-Smad2/3 was subjected to amplification and inserted into the vector, thereby constructing luciferase reporter plasmids based on the manufacturer's instructions (Promega Corporation). A 7-bp mutant vector was also generated from the sites of perfect complementarity. WT and mutant Mut inserts were confirmed by sequencing. A total of 200 ng of WT or Mut reporter vectors were transfected into BEAS-2B cells. The relative luciferase activity (ratio of *Renilla* luciferase activity to that of firefly luciferase) was detected after transfection for 48 h. An equal amount of negative control (NC) mimic was transfected into cells.

Statistical analysis

ORIGIN 9.0 (OriginLab) was used for data analysis. GraphPad Prism 5 (GraphPad Software Inc.) was also used for preliminary data analysis. Data were presented as the mean $\pm$ SD obtained from at least three separate assays. One-way ANOVA and Tukey's post hoc test were used for statistical analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of NR1 on the morphology and migration ability of BEAS-2B cells

Light microscopy was performed to observe the different morphology of BEAS-2B cells following treatment of different concentrations of NR1. BEAS-2B cells were transformed from round to spindle cells and showed significant migration activity in EMT. As shown in Fig. 1A, different concentrations of NR1 significantly influenced the morphology of BEAS-2B cells. Subsequently, 2.5% CSE was applied to induce a COPD model of BEAS-2B cells, and the most effective intervention time on the migration of BEAS-2B cells (24 h; Fig. 1C) and the optimal dose (25 μ g/ml) of NR1 (Fig. 1B) was determined.

Nr1 Alleviates Emt Of Beas-2b Cells Induced By Cse

The most common risk factor for COPD is cigarette smoking. To identify whether CSE causes EMT *in vitro*, the mRNA and protein expression of EMT markers were evaluated via RT-qPCR (Fig. 2A), western blotting (Fig. 2B) and immunofluorescence analysis (Fig. 2C). The data showed that compared with the NC group, CSE reduced the mRNA and protein expression of E-cadherin and increased TGF- β 1, N-cadherin and α -SMA protein as well as mRNA levels to a certain degree (Fig. 2). However, NR1 (25 μ g/ml) reduced TGF- β 1, N-cadherin and α -SMA protein and mRNA expressions and increased the protein and mRNA expression of E-cadherin (Fig. 2). The results indicated that NR1 relieves EMT caused by CSE *in vitro*.

Effects Of Nr1 On Tgf- β 1/smad

Given that TGF- β 1 is a key player in driving EMT, the effects of NR1 on TGF- β 1/Smad pathway components were assessed. Compared with the NC group, CSE increased p-Smad2/3 levels and decreased Smad7 protein expression determined as determined by western blotting. However, NR1 (25 μ g/ml) reduced p-Smad2/3 levels and increased Smad7 protein expression as determined by western blotting (Fig. 3). These results suggested that NR1 decreased the phosphorylation levels of Smad2/3 proteins, which are associated with suppressing the TGF- β 1/Smad pathway.

Nr1 Suppresses Cse-induced P-smad2/3 Nuclear Translocation In Beas-2b Cells

Smad2/3 is a nuclear transcription factor, which requires nuclear translocation to exert its biological function. Hence, the present study determined whether NR1 inhibited p-Smad2/3 nuclear translocation. Western blotting was conducted to determine the nuclear translocation of p-Smad2/3. CSE induced p-Smad2/3 translocation from the cytoplasm into the nucleus; however, NR1 induced the translocation of p-Smad2/3 from the nucleus into the cytoplasm of BEAS-2B cells (Fig. 4). Moreover, signaling mediated by TGF- β is transduced by Smad proteins, and Smad-binding CAGA sequences can be found in the PAI-1 promoter[18]. According to further Dual-Glo-luciferase analysis, NR1 significantly inhibited pGL3-Basic-PAI-1-luc activity enhanced by p-Smad2/3 in cells, consistent with the findings that NR1 inhibited the mRNA and protein expression of p-Smad2/3 (Fig. 5). Overall, the results indicated that NR1 decreased p-Smad2/3 levels to inhibit nuclear translocation of p-Smad2/3 in BEAS-2B cells.

Schematic Of Nr1 Inhibiting Emt Via Tgf- β 1/smad In A Cse-exposed Model

As shown in Fig. 6, in a CSE-exposed model, activated NR1 directly or indirectly reduced the phosphorylation of Smad2/3, and then less p-Smad2/3 translocated into the cytoplasm from the nucleus. Additionally, NR1 treatment further inhibited the TGF- β 1/Smad pathway, thereby improving airway remodeling in COPD. Overall, NR1 relieves the progression of EMT in cells exposed to CSE, whose mechanism may be the inhibition of the TGF- β 1/Smad signaling pathway.

Discussion

COPD is concerning due to its high morbidity rate, and its high burden associated with smoking has been enhanced globally[19]. According to a review and meta-analysis, the morbidity of COPD is much higher in smokers compared with in non-smokers across 28 countries in 1990–2004, and 50% of long-term smokers are affected by COPD[20, 21]. The present study hypothesized that smoking leads to structural changes in the airways and initiates airway remodeling during COPD progression, which is a pathological feature of COPD[4, 22]. Moreover, increasing evidence has shown that EMT may cause airway remodeling; hence, there is a close association between EMT and lung function[4, 5]. The protective effects of NR1 on lung function have been previously reported[23, 24]. In order to have a better

understanding of NR1 on airway remodeling, a CSE-induced COPD model of BEAS-2B cells was treated with NR1. In the present study, NR1 reduced α -SMA expression and recovered decreased E-cadherin expression. It can be observed that NR1 has an inhibitory effect on EMT. EMT is an important process in wound healing and tissue repair, which, however, can also cause complete tissue fibrosis in the case of damage or inflammation, which has been shown to occur in the liver, kidney, and bowel[25–27]. Recent studies found that EMT is a player in balancing the response of the airway to injury and stress and is also involved in the pathogenesis of COPD and asthma[28–30]. Mahmood *et al.*[29] showed that BEAS-2B cells treated with CSE for 24 h enhanced the RNA and protein expression of α -SMA and reduced the RNA and protein levels of E-cadherin, which could be reversed by NR1. To summarize, NR1 treatment suppressed pulmonary EMT induced by CSE in BEAS-2B cells.

TGF- β 1, an important profibrotic cytokine, is widely involved in the onset of airway repair and remodeling[6, 8]. TGF- β 1 facilitates collagen deposition, airway smooth muscle cells, fibroblast proliferation and EMT[31]. TGF- β exerts numerous biological effects via Smad-dependent and -independent pathways[7]. In COPD, the TGF- β 1/Smad pathway is activated by various factors[32–34]. A previous study showed that TGF- β is highly expressed, and its downstream signature protein p-Smad2 is upregulated in rats exposed to cigarette smoke, with increased collagen deposition[35]. The present study found that TGF- β 1 and p-Smad2/3 levels decreased in cells with CSE, indicating that smoking may activate the TGF- β 1/Smad pathway. NR1 protects the heart from ischemia/reperfusion injury, and the possible reason is that it inhibits the TGF- β 1 activation and relieves apoptotic stress[17]. In the present study, TGF- β 1 levels were decreased, and phosphorylation of Smad2/3 was suppressed by NR1 treatment, while Smad7 was increased in cells with CSE. Moreover, the nuclear translocation of Smad2/3 was further influenced by NR1 treatment, thus affecting the TGF- β 1/Smad pathway. NR1 may improve airway remodeling in COPD via inhibiting the TGF- β 1/Smad pathway.

In conclusion, NR1 relieves the progression of EMT in BEAS-2B cells with CSE, which is partly due to its suppression of the TGF- β 1/Smad pathway. However, the exact mechanism of the benefits of NR1 requires further studies. NR1 may serve as a promising target for intervention in airway construction in COPD.

Declarations

Conflicts of interest

The authors state that there are no conflicts of interest.

Consent for publication

The authors give consent to the publication in the journal.

Availability of data and material

All data generated or analysed during this study are included in this published article.

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Authors' contributions

Hui Bi conceived and designed the study. Gui Wang, Zhiying Li conducted most of the experiments. Gui Wang and Zhiying Li analyzed the data. Gui Wang performed the literature search and data extraction. Hui Bi drafted the manuscript. Hui Bi and Tingzan Yan finalized the manuscript. All authors read and approved the final manuscript.

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Figures

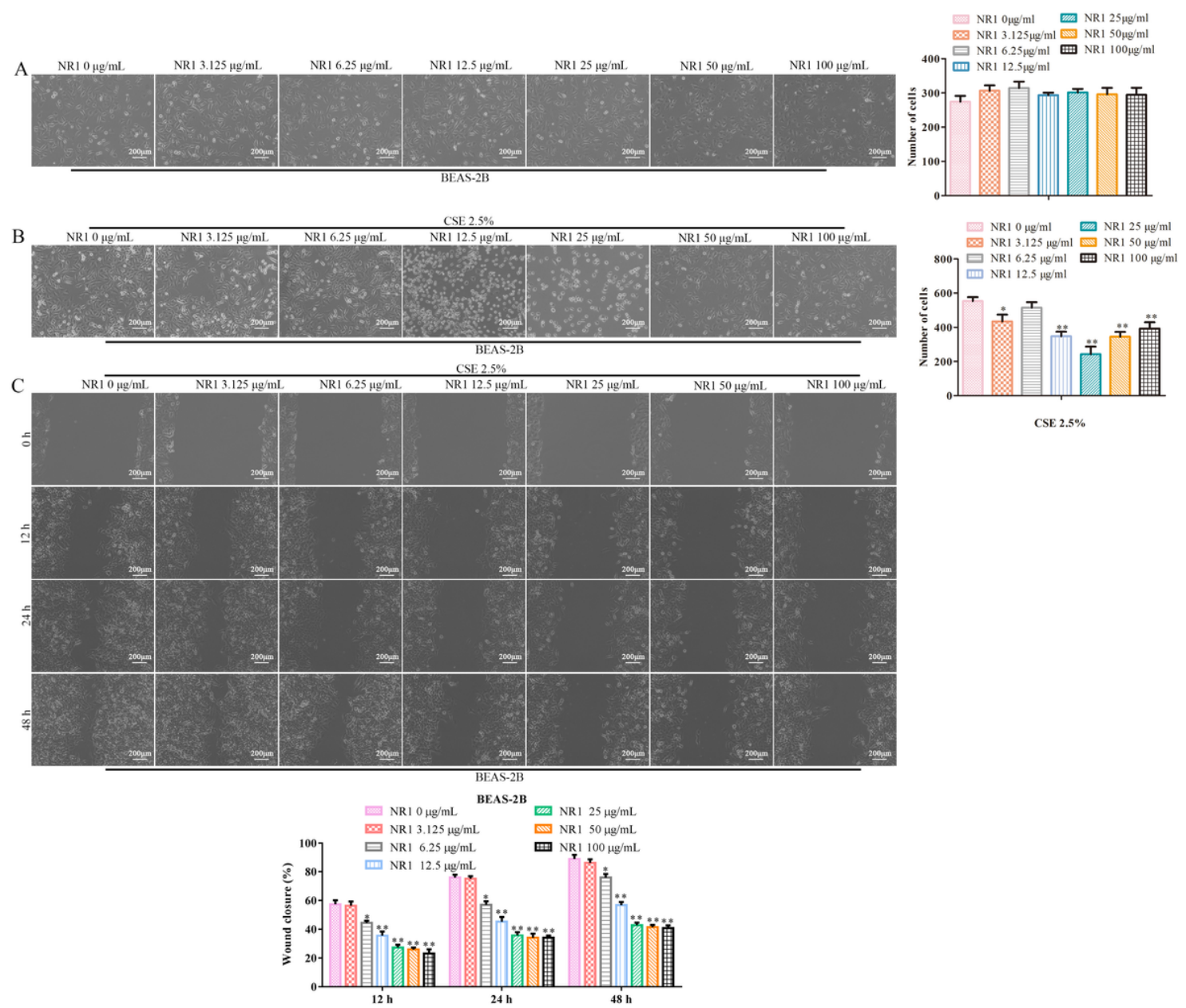


Figure 1

Effects of NR1 on the morphology and migration ability of BEAS-2B cells. (A) Light microscopy of BEAS-2B cells treated with NR1 (magnification, x100). (B) Light microscopy of BEAS-2B cells induced with 2.5% CSE and treated with NR1 (magnification, x100). (C) Wound healing assay analysis of the migration ability of BEAS-2B cells induced with 2.5% CSE and treated with NR1. All values were expressed as the mean $\pm$ standard deviation of at least three assays. * $P < 0.05$ and ** $P < 0.01$ vs. NC. NR1, notoginsenoside R1; CSE; cigarette smoke extract; NC, negative control.

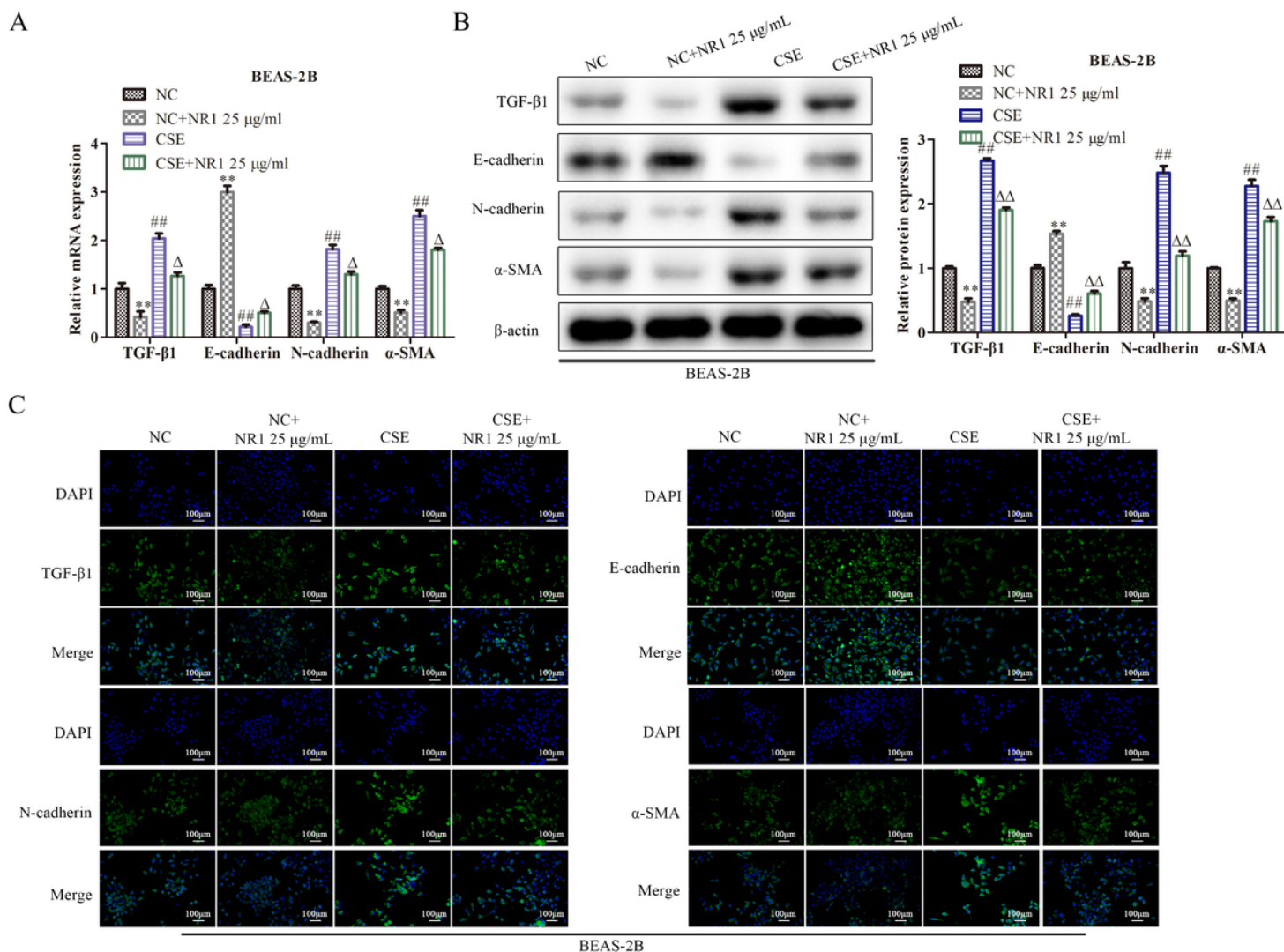


Figure 2

NR1 alleviates epithelial-mesenchymal transition of BEAS-2B cells induced by CSE. (A) Reverse transcription-quantitative PCR results of the RNA expression of TGF- β 1, E-cadherin, N-cadherin and α -SMA. (B) Western blot assay and (C) immunofluorescence assay analysis of TGF- β 1, E-cadherin, N-cadherin and α -SMA protein expression. All values were presented as the mean $\pm$ standard deviation of at least three assays. One-way ANOVA and Tukey's post hoc test were used to determine statistical significance. ** $P < 0.01$ vs. NC group. ## $P < 0.01$ vs. CSE group. Δ $P < 0.05$ and $\Delta\Delta$ $P < 0.01$ vs. CSE + NR1 group. NR1, notoginsenoside R1; CSE; cigarette smoke extract; NC, negative control; α -SMA; α -smooth muscle actin.

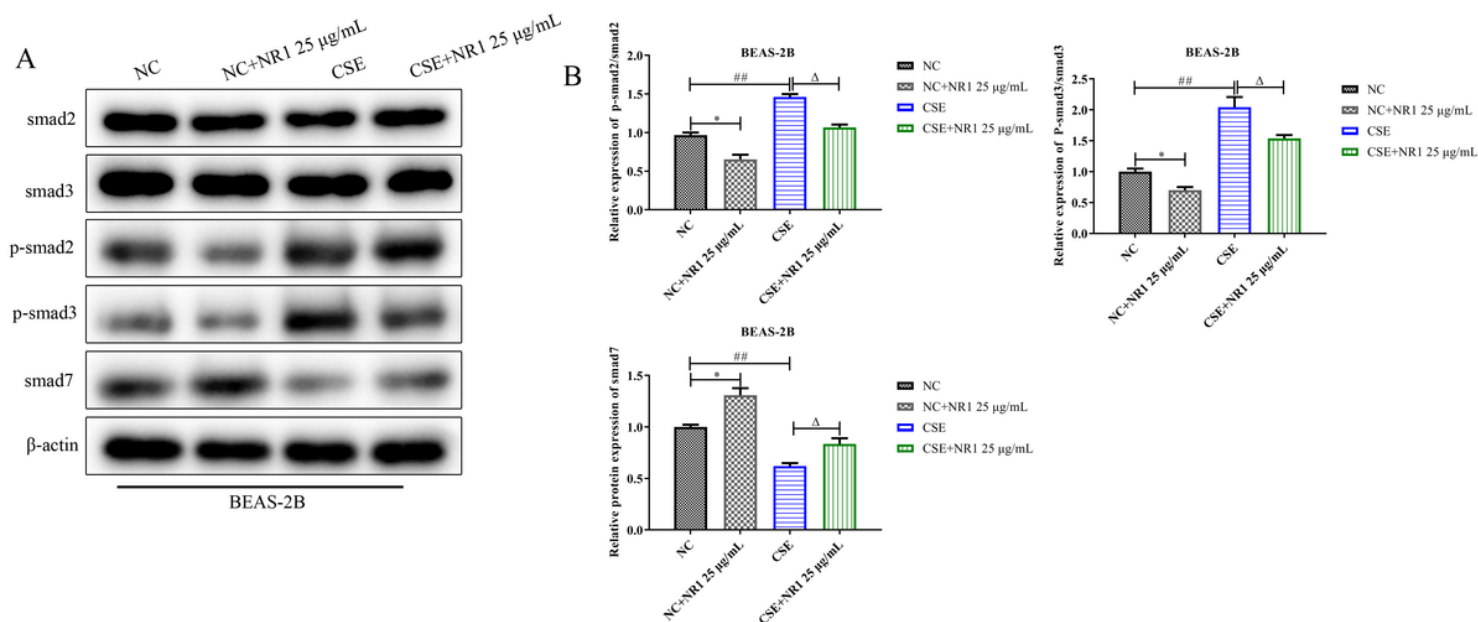


Figure 3

Effects of NR1 on the TGF- β 1/Smad signaling pathway. (A and B) Analysis of p-Smad2, total Smad2, p-Smad3, total Smad3 and total Smad7 levels using western blotting. All values were presented as the mean $\pm$ standard deviation of at least three assays. One-way ANOVA and Tukey's post hoc test were used to determine statistical significance. $**P < 0.01$ vs. NC group. $##P < 0.01$ vs. CSE group. $\Delta P < 0.05$ and $\Delta\Delta P < 0.01$ vs. CSE + NR1 group. NR1, notoginsenoside R1; p, phosphorylated; CSE; cigarette smoke extract; NC, negative control.

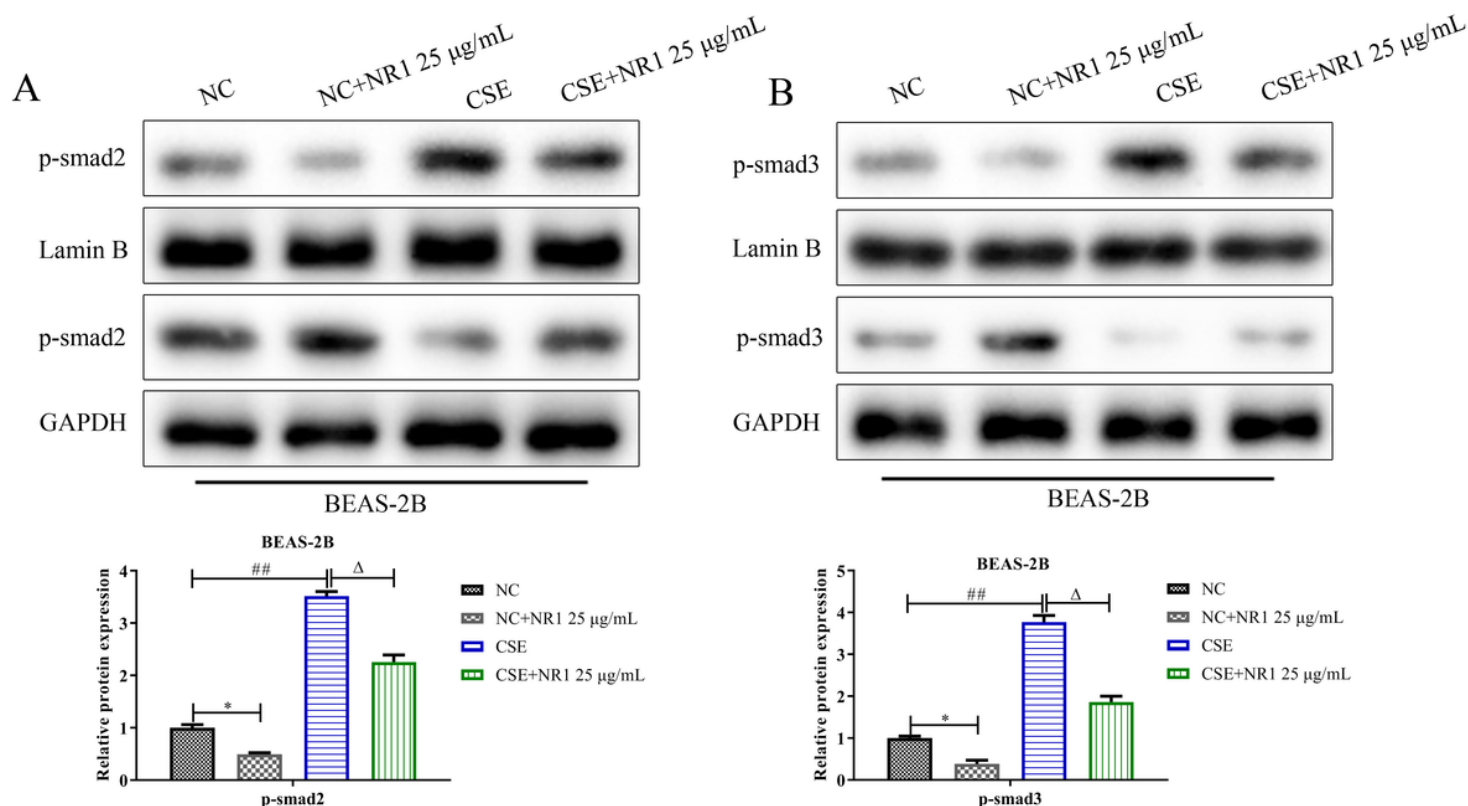


Figure 4

NR1 suppresses CSE-induced nuclear translocation of p-Smad2/3 in BEAS-2B cells. (A and B) Analysis of p-Smad2/3 levels in the nucleus and cytoplasm using western blotting. All values were presented as the mean $\pm$ standard deviation of at least three assays. One-way ANOVA and Tukey's post hoc test were used to determine statistical significance. $** P < 0.01$ vs. NC group. $## P < 0.01$ vs. CSE group. $\Delta P < 0.05$ and $\Delta\Delta P < 0.01$ vs. CSE + NR1 group. NR1, notoginsenoside R1; p, phosphorylated; CSE; cigarette smoke extract; NC, negative control.

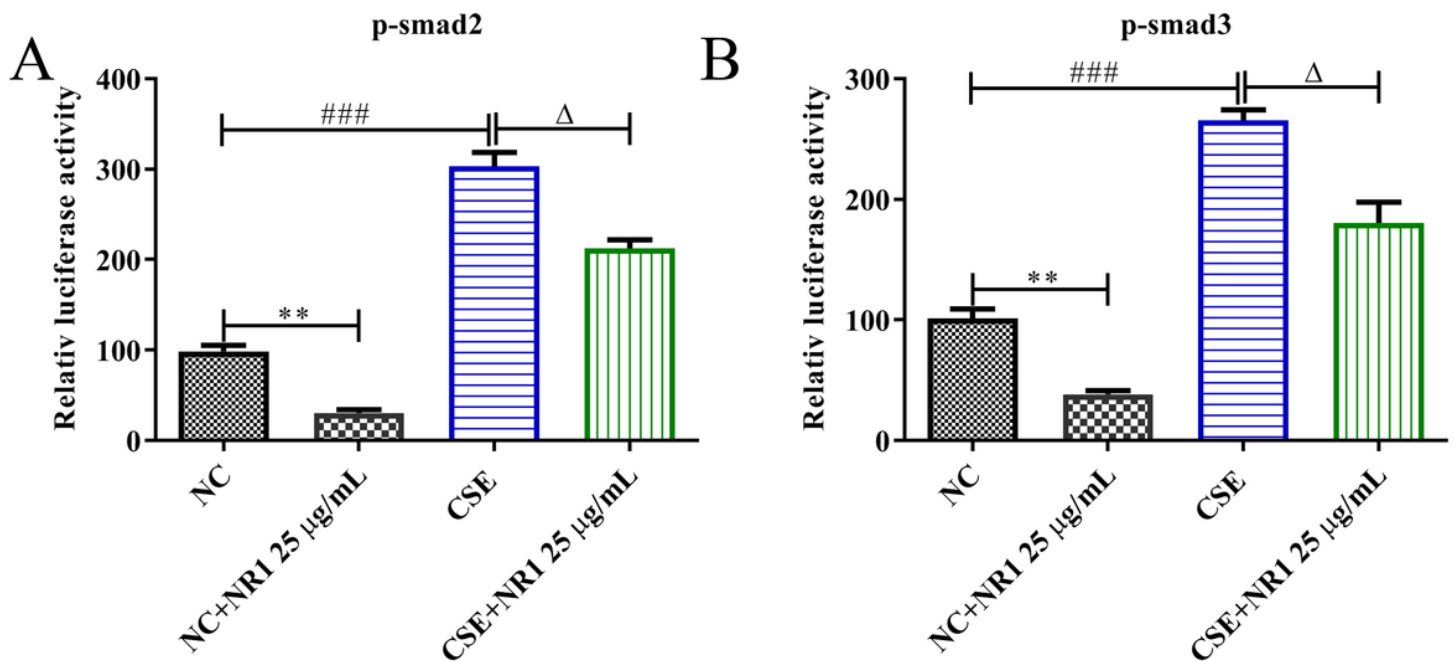


Figure 5

NR1 further inhibits the binding activity of p-Smad2 and p-Smad3 to PAI-1. (A and B) Dual-Glo-luciferase analysis demonstrated the binding activity of p-Smad2/3 to PAI-1. All values are presented as the mean $\pm$ standard deviation of at least three assays. One-way ANOVA and Tukey's post hoc test were used to determine statistical significance. $** P < 0.01$ vs. NC group. $## P < 0.01$ vs. CSE group. $\Delta P < 0.05$ and $\Delta\Delta P < 0.01$ vs. CSE + NR1 group. NR1, notoginsenoside R1; p, phosphorylated; CSE; cigarette smoke extract; NC, negative control; PAI,1, plasminogen activator inhibitor 1.