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Original Article

Tetrandrine suppresses the TGF-β1-induced proliferation and migration of airway smooth muscle cells using the Nrf2-ARE signaling pathway

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Abstract: Airway smooth muscle cell (ASMCs) proliferation and migration facilitates airway remodeling in asthma. Tetrandrine is a major compound derived from *Stephania tetrandra* S. Moore that has anti-allergic activity. This study aimed to investigate the effect of tetrandrine on ASMCs' proliferation and migration and its underlying mechanism. Transforming growth factor-β1 (TGF-β1)-treated ASMCs were cultured for analyses *in vitro*. Cell proliferation was measured using an MTT assay. Cytotoxicity was investigated using lactate dehydrogenase (LDH) release and apoptosis. The cell cycle distribution was determined using flow cytometry. Cell migration was measured using a trans-well assay. The levels of reactive oxygen species (ROS) production, superoxide dismutase (SOD) activity, glutathione (GSH) activity, and malonaldehyde (MDA) content were quantified using a specific kit. The levels of the extracellular matrix (ECM)-related proteins were detected by western blot. The nuclear factor E2-related factor 2 (Nrf2)-antioxidant response element (ARE) pathway was evaluated using western blot. Tetrandrine inhibited TGF-β1-caused proliferation and migration in ASMCs. Tetrandrine regulated the cell cycle distribution but did not affect the LDH release or the apoptosis upon the stimulation of TGF-β1. Tetrandrine suppressed TGF-β1-induced oxidant stress and ECM in the ASMCs. Tetrandrine promoted the Nrf2-ARE pathway, and the knockdown of Nrf2 weakened the function of tetrandrine on the proliferation, migration, oxidant stress, and ECM. In conclusion, Tetrandrine represses proliferation and migration in TGF-β1-treated ASMCs by inhibiting oxidant stress and ECM using the Nrf2-ARE pathway, indicating a novel avenue for the therapy of asthma airway remodeling.

Keywords: Asthma airway remodeling, tetrandrine, Nrf2, ASMCs, proliferation, migration

Introduction

Asthma is a common respiratory disease, which is characterized by airway inflammation and remodeling [1]. Airway remodeling is characterized by airway wall thickening and an increase in smooth muscle mass [2]. The proliferation and migration of airway smooth muscle cells (ASMCs) are important factors during asthma [3]. Thus, therapeutic treatment by the reduction of ASMC proliferation and migration may be useful for preventing the development of asthma.

The Chinese herbal medicine and its derived compounds have been reported to have an important impact on the outcome of asthma [4, 5]. Tetrandrine is a major compound derived from the roots of *Stephania tetrandra* S. Moore, which exhibits pharmacological property in cancers and diseases by regulating reactive oxygen species (ROS), autophagy, drug resistance, and the cell cycle process [6]. For example, one study found that tetrandrine effectively inhibits tumor malignancy in gliomas [7]. The emerging evidence suggests that tetrandrine induces autophagy and suppresses cell proliferation in triple-negative breast cancer [8]. Moreover, tetrandrine is regarded as a protective agent for ischemic stroke injury [9]. More importantly, tetrandrine plays promising pharmacological actions in inflammatory pulmonary diseases, including asthma [10]. However, the effect and mechanism of tetrandrine on ASMCs' proliferation and migration remain largely unclear.

The nuclear factor E2-related factor 2 (Nrf2) signaling exhibits an essential function in pre-

venting oxidant stress-induced injury in respiratory diseases [11]. Moreover, Nrf2 plays an important protective role in airways [12]. Nrf2 works as a key factor against oxidant stress in asthma by regulating the antioxidant response element (ARE), including NAD(P)H: quinone oxidoreductase (NQO1), and heme oxygenase-1 (HO-1) [13, 14]. A previous study suggests that the activation of Nrf2 suppresses ASMCs' proliferation [14]. Hence, the hypothesis was formed that tetrandrine might regulate asthma airway remodeling using the Nrf2-ARE pathway. Transforming growth factor-β1 (TGF-β1) is a vital regulator that participates in the progression of lung diseases, including asthma, and it has been widely used *in vitro* as a model of asthma airway remodeling [15-17]. In an attempt to evaluate the role and mechanisms of tetrandrine in asthma airway remodeling, using TGF-β1-treated ASMCs, this study focused on the effect of tetrandrine on cell proliferation and migration and investigated the underlying mechanism.

Materials and methods

Cell culture and treatment

ASMCs were harvested from patients without asthma at Jinhua Polytechnic. A written informed consent was provided by each participant, and the study procedures were approved by the ethics committee of Jinhua Polytechnic. The primary ASMCs were prepared as previously reported [18]. The cells were maintained in DMEM (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum at 37°C under 5% CO_2. The ASMCs were stimulated using 10 ng/ml TGF-β1 (BioLegend, San Diego, CA, USA) for the indicated time, and an untreated group was incubated with an equal volume of PBS solution. To investigate the effect of tetrandrine, the cells were exposed to the indicated concentration of tetrandrine (Selleck, Shanghai, China) for 30 min and the cells stimulated with DMSO (Sigma, St. Louis, MO, USA) were regarded as a control. For the knockdown of Nrf2, a small interfering RNA against Nrf2 (si-Nrf2) obtained from Genepharma (Shanghai, China) was transfected into the ASMCs using the Lipofectamine™ 2000 Reagent (Thermo Fisher Scientific, Waltham, MA, USA). After 24 h, the cells were collected for further treatment with tetrandrine or TGF-β1.

Cell proliferation

Cell proliferation was examined using MTT (Genechem, Shanghai, China). ASMCs were seeded into 96-well plates in quadruplicate at a density of 1×10^4 cells per well overnight, then pre-treated with different concentrations (0, 1, 2, and 4 μM) of tetrandrine for 30 min and incubated with 10 ng/ml TGF-β1 for different time points (0, 24, 48 and 72 h). At the end point, the cells were interacted with 0.5 mg/ml of MTT solution for 4 h. Subsequently, formazan was dissolved using DMSO. The absorbance at 490 nm was determined using a microplate reader (Bio-Rad, Hercules, CA, USA).

Lactate dehydrogenase (LDH) release assay

The cytotoxicity was analyzed via an LDH release using an LDH cytotoxicity assay kit (Beyotime, Shanghai, China). ASMCs (1×10^4 cells/well) were seeded into 96-well plates. After the indicated treatment, the LDH release in the cell culture medium and the total LDH in the cells were measured following the manufacturer's instructions. The absorbance at 490 nm was determined using a microplate reader.

Cell apoptosis and cell cycle

Flow cytometry was exploited to assess cell apoptosis and cell cycle distribution. For the apoptosis assay, ASMCs were exposed to the indicated concentration of tetrandrine and TGF-β1, followed by collection for further analysis using an Annexin V-FITC/PI apoptosis detection kit (Vazyme, Nanjing, China) according to the instructions. The apoptotic rate was analyzed via a flow cytometer (BD Biosciences, San Jose, CA, USA).

For the cell cycle assay, the treated cells were fixed with ethanol (Sigma) and stained with PI/RNase A staining solution (Sigma). The stained cells were analyzed using a flow cytometer.

Western blot

After the treatment of tetrandrine and TGF-β1, the ASMCs were lysed using a RIPA buffer (Yeasen, Shanghai, China). The concentration of protein samples was determined using a BCA Protein Assay Kit (Beyotime). The denaturized samples (20 μg) were subject to SDS-PAGE and membrane transfer with Immobilon-PVDF

transfer membranes (Solarbio, Beijing, China). After being blocked with a 5% BSA blocking buffer (Solarbio), the membranes were incubated with the primary antibodies against cyclin D1 (ab226977), $p21^{Cip1}$ (ab227443), E-cadherin (ab15148), Vimentin (ab92547), alpha smooth muscle actin (α-SMA) (ab32575), collagen I (ab138492), collagen III (ab184993), Nrf2 (ab62352), NQO1 (ab97385), HO-1 (ab13243), or GAPDH (ab181602), and a corresponding secondary antibody (ab6721), which were purchased form Abcam (Cambridge, MA, USA). The Super ECL Detection Kit (Keygen Biotech, Nanjing, China) was used for the visualization of the protein signals. Image Lab software (Bio-Rad) was used for the densitometry analysis and the relative expression of protein was expressed as the ratio of the indicated protein and GAPDH.

Trans-well migration assay

A trans-well migration assay was conducted with 24-well trans-well chambers. Briefly, ASMCs (1×10^4) were re-suspended in a serum-free medium and placed into the upper chambers. 600 μl of a medium containing 10% fetal bovine serum with different concentrations (0, 1, 2 and 4 μM) of tetrandrine or 10 ng/ml TGF-β1 was added to the lower chamber. Following being cultured for 24 h at 37°C, the migrated cells were stained with 0.1% crystal violet (Sigma). With three random visual fields, the stained cells were photographed and counted under a microscope (Olympus, Tokyo, Japan).

Oxidant stress assays

For the intracellular ROS assay, the ASMCs were seeded into 96 well plates (1×10^4 cells/per well) and then exposed to different concentrations (0, 1, 2 and 4 μM) of tetrandrine for 30 min. Subsequently, the cells were stimulated using 10 ng/ml TGF-β1 for 24 h and washed with PBS for the DCFH-DA staining using a Reactive Oxygen Species Assay Kit (Beyotime) following the manufacturer's instructions. The ROS fluorescence intensity was measured using a fluorescent microplate reader (Thermo Fisher).

For the activities of the anti-oxidative enzymes (superoxide dismutase [SOD] and glutathione [GSH]), the treated ASMCs were harvested and lysed for the activity measurement using a commercial kit (Beyotime). The cellular SOD and GSH activities were expressed as units per mg total protein.

For the measurement of malonaldehyde (MDA) content in the cell culture medium, the medium was collected after the indicated treatment. The level of MDA was determined using a commercial MDA assay kit (Beyotime) following the kit's instructions.

Statistical analysis

The experiments were repeated 3 times. The data were expressed as the means ± standard errors of the mean (SEM) via GraphPad Prism 7 software (GraphPad Inc., La Jolla, CA, USA). Statistical significance was assessed via Student's *t*-test or ANOVA. $P < 0.05$ was regarded as statistically significant.

Results

Tetrandrine inhibits proliferation and migration in TGF-β1-treated ASMCs

To explore the role of tetrandrine in asthma airway remodeling, the ASMCs were treated with tetrandrine and TGF-β1. As described in **Figure 1A**, exposure to TGF-β1 significantly enhanced cell proliferation compared with the untreated group at 24, 48 and 72 h, but this effect was dose dependently inhibited by the treatment with tetrandrine. However, the treatment with tetrandrine and TGF-β1 showed little effect on the LDH release or cell apoptosis at 24 h, suggesting that the effect of the proliferation was not induced by cytotoxicity (**Figure 1B**, **1C**). Meanwhile, the stimulation of TGF-β1 markedly reduced the proportion of ASMCs at the G0/G1 phase, which were progressively counteracted by the introduction of tetrandrine (**Figure 1D**). Similarly, the western blot data revealed that the protein expression of cyclin D1, a transition promoter of the S phase, was clearly elevated in the ASMCs by the treatment with TGF-β1 but decreased by the treatment with tetrandrine, and the G1 phase gatekeeper $p21^{Cip1}$ protein level showed an opposite trend (**Figure 1E**). Furthermore, the results of the trans-well assay showed that the number of migrative cells was conspicuously enhanced in the ASMCs after the challenge of TGF-β1, but this event was weakened by the treatment of tetrandrine in a concentration-dependent manner (**Figure 1F**). These results showed that tetrandrine suppressed the proliferation and migration of TGF-β1-treated ASMCs.

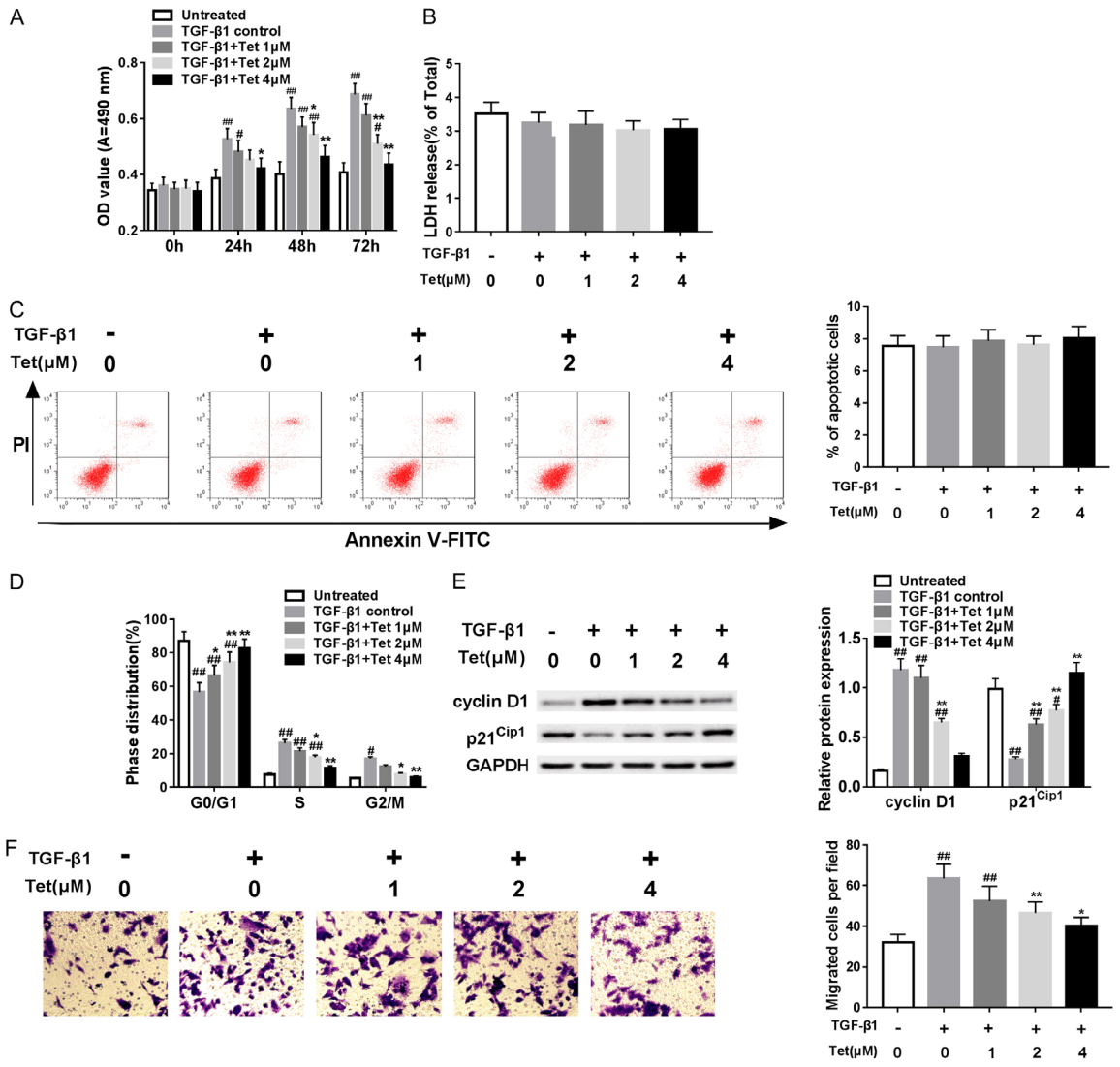

Figure 1. Tetrandrine inhibits TGF-β1-induced proliferation and migration in ASMCs. (A) Cell proliferation was measured in the ASMCs after treatment with tetrandrine and TGF-β1 using an MTT assay. LDH release (B), apoptosis (C), cell cycle (D and E) and migration (F) were quantified in the ASMCs after treatment with tetrandrine for 30 min and TGF-β1 for 24 h. *$P < 0.05$, **$P < 0.01$, versus the control group (without treatment with tetrandrine); #$P < 0.05$, ##$P < 0.01$, versus the untreated group (without treatment with TGF-β1).

Tetrandrine decreases oxidant stress and ECM in TGF-β1-treated ASMCs

To explore the potential mechanism that allows tetrandrine to participate in the migration and invasion of ASMCs, its effect on oxidant stress and the extracellular matrix (ECM) was investigated in TGF-β1-treated ASMCs. ASMCs were pre-treated with different concentrations (0, 1, 2 and 4 μM) of tetrandrine for 30 min before the stimulation of 10 ng/ml of TGF-β1 for 24 h. As shown in **Figure 2A-D**, the treatment with TGF-β1 significantly increased the ROS fluorescence intensity and the MDA content but decreased the activities of SOD and GSH, indicating that TGF-β1 induced oxidant stress in the ASMCs. However, this event was progressively alleviated by the treatment of tetrandrine. In addition, the expression levels of the ECM-related proteins were measured in the treated cells. The results displayed in **Figure 2E**, **2F** show that the level of E-cadherin protein was notably decreased by TGF-β1, which was dose dependently restored by the treatment with tetrandrine. However, the protein abundances of vimentin, α-SMA, and collagen I and III were evidently increased by the stimulation of TGF-β1, and were mitigated by the introduction of tet-

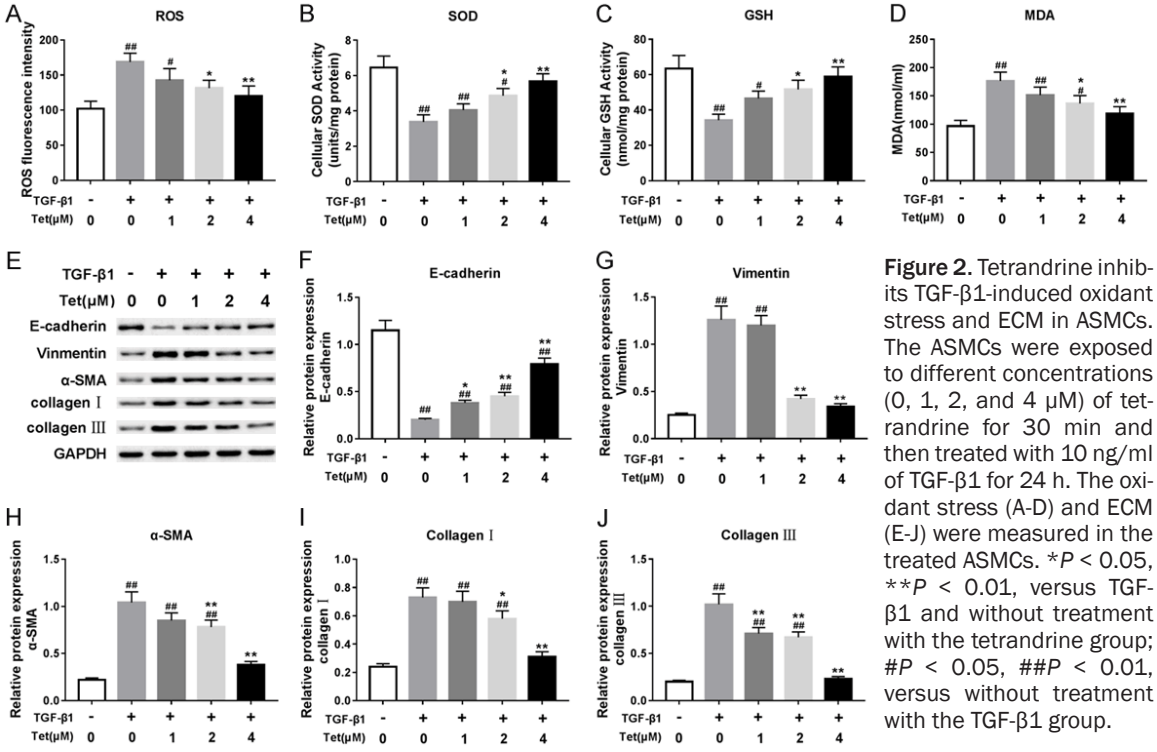

Figure 2. Tetrandrine inhibits its TGF-β1-induced oxidant stress and ECM in ASMCs. The ASMCs were exposed to different concentrations (0, 1, 2, and 4 μM) of tetrandrine for 30 min and then treated with 10 ng/ml of TGF-β1 for 24 h. The oxidant stress (A-D) and ECM (E-J) were measured in the treated ASMCs. **P* < 0.05, ***P* < 0.01, versus TGF-β1 and without treatment with the tetrandrine group; #*P* < 0.05, ##*P* < 0.01, versus without treatment with the TGF-β1 group.

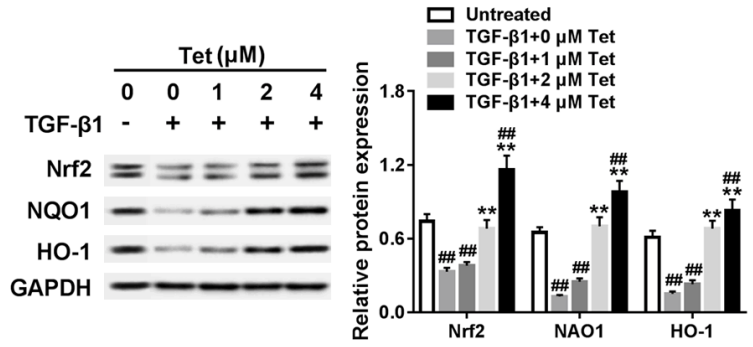

Figure 3. Tetrandrine promotes the Nrf2-ARE pathway. The expression levels of Nrf2, NQO1, and HO-1 were measured in the ASMCs after treatment with different concentrations (0, 1, 2, and 4 μM) of tetrandrine and 10 ng/ml of TGF-β1 using western blot. ***P* < 0.01, versus TGF-β1 control (without treatment with the tetrandrine group); ##*P* < 0.01, versus the untreated group (without treatment of TGF-β1).

randrine (**Figure 2E**, **2G-J**). These data suggest that tetrandrine represses oxidant stress and ECM in TGF-β1-treated ASMCs.

Tetrandrine inhibits proliferation and migration by activating the Nrf2-ARE pathway in TGF-β1-treated ASMCs

Given that Nrf2 is an important antioxidant, the effect of tetrandrine on the Nrf2-ARE pathway was investigated in TGF-β1-treated ASMCs. As displayed in **Figure 3**, the protein levels of Nrf2, NQO1 and HO-1 were significantly decreased by the stimulation of TGF-β1, but progressively elevated by the treatment with tetrandrine in a concentration-dependent manner. To explore whether Nrf2 was involved in the tetrandrine-mediated mechanisms, the abundance of Nrf2 was knocked down using si-Nrf2 in ASMCs before treatment with tetrandrine and TGF-β1. At 72 h, cell proliferation was significantly promoted by the knockdown of Nrf2 in TGF-β1-challenged ASMCs with or without the treatment of tetrandrine compared with their corresponding control (**Figure 4A**). However, the knockdown of Nrf2 could not affect the LDH release and apoptosis, which was not statistically significant among the groups (**Figure 4B**, **4C**). Additionally, the interference of Nrf2 effectively reduced the distribution of cells at the G0/G1 phase in the TGF-β1-challenged ASMCs, but this effect was reversed by the introduction of tetrandrine (**Figure 4D**, **4E**). Furthermore, silencing Nrf2 promoted TGF-β1-induced migration in the ASMCs with or without treatment with tetran-

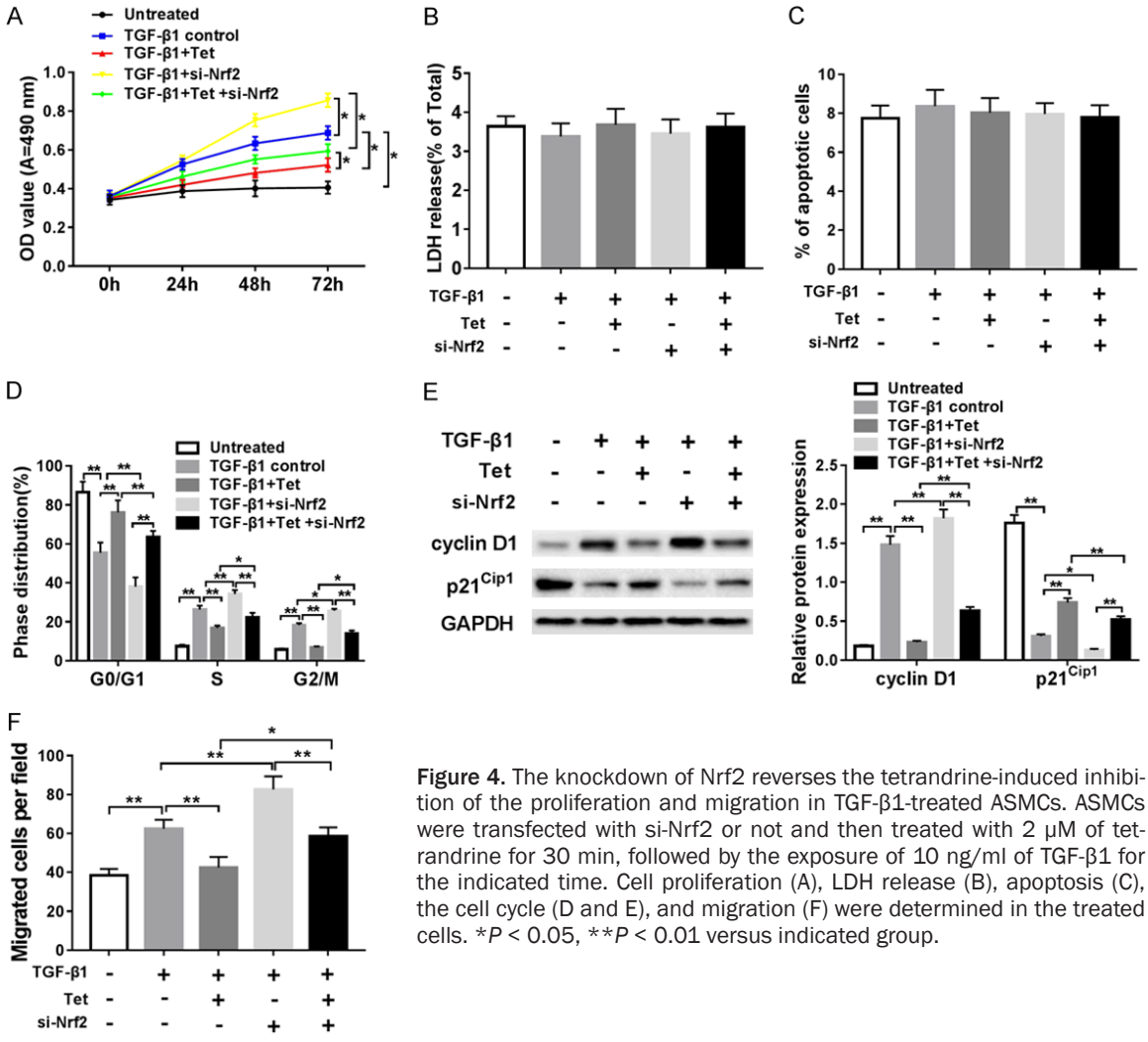

Figure 4. The knockdown of Nrf2 reverses the tetrandrine-induced inhibition of the proliferation and migration in TGF-β1-treated ASMCs. ASMCs were transfected with si-Nrf2 or not and then treated with 2 μM of tetrandrine for 30 min, followed by the exposure of 10 ng/ml of TGF-β1 for the indicated time. Cell proliferation (A), LDH release (B), apoptosis (C), the cell cycle (D and E), and migration (F) were determined in the treated cells. *$P < 0.05$, **$P < 0.01$ versus indicated group.

drine (**Figure 4F**). In addition, the knockdown of Nrf2 aggravated TGF-β1-induced oxidant stress and attenuated the suppressive role of tetrandrine in oxidant stress (**Figure 5A-D**). In addition, the Nrf2 silencing contributed to ECM protein expression in TGF-β1-challenged ASMCs with or without treatment with tetrandrine (**Figure 5E-J**). These findings indicated that tetrandrine suppresses proliferation and migration by regulating the Nrf2-ARE pathway in TGF-β1-treated ASMCs.

Discussion

The proliferation and migration of ASMCs contribute to the increase of airway smooth muscle thickness and asthma airway remodeling [19]. In our research, ASMCs were challenged by TGF-β1 to induce cell proliferation and migration, possibly leading to asthma airway remodeling. Moreover, this research demonstrated that tetrandrine re-activated the TGF-β1-blocked Nrf2-ARE pathway to decrease oxidant stress and ECM, leading to the inhibition of ASMCs' proliferation and migration, which might delay asthma airway remodeling.

A previous study revealed the anti-proliferation and anti-metastasis roles of tetrandrine in human cells [20, 21]. This study found that tetrandrine decreased ASMCs' proliferation and migration induced by TGF-β1. Furthermore, using LDH release assay and flow cytometry, we found that the anti-proliferation role of tetrandrine was realized by regulating the cell cycle process but not by cytotoxicity, which is also in agreement with the data in previous works [22, 23]. However, the mechanism by which tetrandrine regulates ASMCs' functions remains unclear. Oxidant stress and ECM are

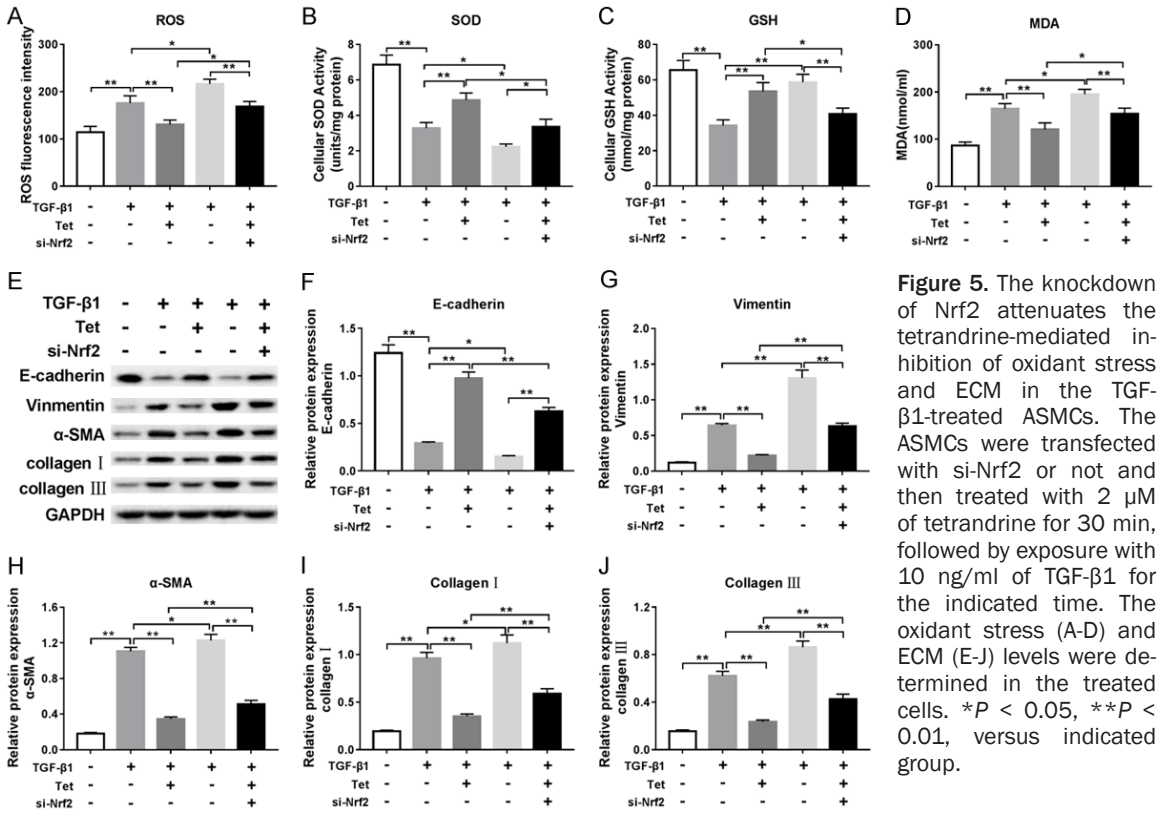

Figure 5. The knockdown of Nrf2 attenuates the tetrandrine-mediated inhibition of oxidant stress and ECM in the TGF-β1-treated ASMCs. The ASMCs were transfected with si-Nrf2 or not and then treated with 2 μM of tetrandrine for 30 min, followed by exposure with 10 ng/ml of TGF-β1 for the indicated time. The oxidant stress (A-D) and ECM (E-J) levels were determined in the treated cells. $*P < 0.05$, $**P < 0.01$, versus indicated group.

the key pathogeneses of asthma airway remodeling [24-26]. Moreover, they are associated with ASMCs' proliferation and migration [14, 18, 27]. Oxidant stress is defined as an out of balance ROS metabolism, and its damage could be ameliorated using Chinese herbal medicine [28, 29]. Previous studies showed that tetrandrine could attenuate hydrogen peroxide or lipopolysaccharide-induced oxidant injury [30, 31]. Consistently with our findings, tetrandrine also decreased TGF-β1-induced oxidative stress, as revealed by the reduction of the oxidants ROS and MDA and the increase of the anti-oxidants SOD and MDA. Furthermore, E-cadherin, vimentin, α-SMA, and collagen I and III are the important factors associated with ECM, which determines airway wall thickness [32]. According to their expression levels, we found that tetrandrine repressed TGF-β1-induced ECM. These results indicated that tetrandrine might suppress proliferation and migration in ASMCs by blocking oxidant stress and ECM.

Nrf2 is promoted as a protective agent in lung injury [33]. In this work, after treatment with TGF-β1, Nrf2 expression was decreased in the ASMCs, which are also in agreement with a former study that showed a low expression of Nrf2 in asthma [34]. Nrf2 activation results in the increased expression of the anti-oxidant proteins NQO1 and HO-1 [35]. Nrf2-mediated anti-oxidant signaling is required for the inhibition of airway remodeling in asthma [36, 37]. Moreover, Nrf2-ARE-mediated anti-oxidant singling could reduce ECM to block fibrosis [38, 39]. Also, Nrf2 is thought to suppress the TGF-β1-induced proliferation and inflammatory response in ASMCs [14]. Studies indicate that the Nrf2-ARE pathway might be involved in asthma airway remodeling by decreasing ASMCs' proliferation and migration by regulating the oxidant response and ECM. Here we found that tetrandrine ablated the suppressive role of TGF-β1 in Nrf2-ARE signaling, indicating the anti-oxidant role of tetrandrine by promoting Nrf2, NQO1, and HO-1 expressions. Additionally, we found that the effect of tetrandrine on the proliferation, migration, oxidant stress, and ECM was counteracted by the silencing of Nrf2 using siRNA, revealing that tetrandrine repressed TGF-β1-caused ASMCs' proliferation and migration by activating Nrf2-ARE pathway.

In conclusion, our study is the first to demonstrate that tetrandrine suppresses ASMCs' proliferation and migration induced by TGF-β1,

which is possibly mediated by the activation of the Nrf2-ARE pathway, thus providing a potential therapeutic treatment for asthma airway remodeling.

Acknowledgements

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Disclosure of conflict of interest

None.

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