



Atractylon Treatment Attenuates Pulmonary Fibrosis via Regulation of the mmu_circ_0000981/miR-211-5p/TGFBR2 Axis in an Ovalbumin-Induced Asthma Mouse Model

Haizhu Zeng^{1,2}, Hongchang Gao,¹ Meilan Zhang,¹ Jinrui Wang,¹ Yuxia Gu,¹ Yumeng Wang,¹ Huali Zhang,¹ Panpan Liu,¹ Xia Zhang,¹ and Lei Zhao^{1,2}

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Abstract—Asthma-induced pulmonary fibrosis (PF) is an important public health concern that has few treatment options given its poorly understood etiology; however, the epithelial to mesenchymal transition (EMT) of pulmonary epithelial cells has been implicated to play an important role in inducing PF. Although previous studies have found atractylon (Atr) to have anti-inflammatory effects, whether Atr has anti-PF abilities remains unknown. The purpose of the current study was to validate the protective efficiency of Atr in both an animal model of ovalbumin (OVA)-induced asthma and an EMT model induced by transforming growth factor- β 1 (TGF- β 1) using TC-1 cells. The results of this study revealed that Atr treatment suppressed OVA-induced PF via fibrosis-related protein expression. Atr treatment suppressed OVA-induced circRNA-0000981 and TGFBR2 expression but promoted miR-211-5p expression. In vivo studies revealed that Atr suppressed TGF- β 1-induced EMT and fibrosis-related protein expression via suppressing circRNA-0000981 and TGFBR2 expression. The results also suggested that the downregulation of circRNA-0000981 expression suppressed TGFBR2 by sponging miR-211-5p, which was validated by a luciferase reporter assay. Collectively, the findings of the present study suggest that Atr treatment attenuates PF by regulating the mmu_circ_0000981/miR-211-5p/TGFBR2 axis in an OVA-induced asthma mouse model.

KEY WORDS: Atractylon; TGFBR2; mmu_circ_0000981; miR-211-5p; asthma.

INTRODUCTION

Asthma is a chronic condition that is typically characterized by airway obstruction, bronchial inflammation, airflow disruption, and airway hyper-responsiveness. Asthma affects approximately 30 million individuals in the USA alone and substantially more around the globe. Since asthma is the most prevalent chronic childhood condition,

¹ Department of Respiratory Medicine, Shanghai Gongli Hospital, 219 Miao-Pu Road, Shanghai, 200135, People's Republic of China

² To whom correspondence should be addressed at Department of Respiratory Medicine, Shanghai Gongli Hospital, 219 Miao-Pu Road, Shanghai, 200135, People's Republic of China. E-mails: kyzhz@126.com; zhaolei7012@126.com

its efficient treatment and control have become crucial for protecting the health of children [1, 2]. Our previous research found that oxidative stress was correlated with DNA damage, which is frequently decreased by the release of ROS from respiratory tract epithelial or neutrophils cells, leading to airway remodeling and subepithelial fibrosis [3]. Additional evidence indicates that the mesenchymal transition (EMT) participates in the progression of pulmonary fibrosis (PF); however, the mechanism remains unclear.

Emerging research has verified that a type of novel non-coding RNA, termed circular RNA (circRNA), participates in multiple pathological and physiological processes [4–6]. CircRNAs are commonly produced by back-splicing from the protein-coding gene exons and constructed by an integration of the 3' and 5' ends. Various studies have been published on the genome-wide expression patterns, regulation, and biogenesis of circRNAs. Nevertheless, the function of circRNAs remains poorly understood [7–9]. MicroRNAs (miRNAs) are small, short, noncoding RNAs that inhibit gene expression by binding to the mRNA 3'-untranslated region (3'-UTR) of the target gene. Moreover, while the function of miRNA has been shown to be regulated by circRNAs [10], the relationship between miRNAs, circRNAs, and asthma remains unclear.

Atractylodes Rhizoma, made from the rhizomes of *Atractylodes japonica* Koidzumi, is used for the treatment of pain, gastritis, and enteritis [11]. The extracts of *Atractylodes* rhizomes also exhibit a significant protective effect in the liver by suppressing hepatic toxicity and tert-butyl hydroperoxide-induced DNA damage in rat hepatocytes [12]. Increasing evidence has revealed that the core element of *Atractylodes* rhizomes has an anti-inflammatory effect [13]. Thus, the purpose of the current investigation was to identify whether atractylon mediates a protective effect in an animal model of OVA-induced asthma and a TGF- β -induced EMT model using TC-1 cells.

MATERIALS AND METHODS

Ethics Statement

The Ethics Committee of Gongli Hospital in Pudong New Area approved the study. Mice were anesthetized via an intraperitoneal injection of sodium pentobarbital (30 mg/kg) prior to euthanasia.

OVA-Induced Asthma Murine Model

Male BALB/c mice aged 6 to 8 weeks weighing 18–24 g were purchased from the SLAC Laboratory Animal

Co., Ltd. (Shanghai, China). We initiated the asthma model by treatment with OVA. We sensitized the mice on days 0, 7, and 14 by an intraperitoneal injection of 20 μ g OVA emulsified in 1 mg aluminum hydroxide in a total volume of 0.2 mL. Seven days after the last sensitization, we exposed the mice to an OVA aerosol of 1% for at most 1 h per day for 1 week. The 1% OVA aerosol was produced by a compressed air atomizer driven by filling a Perspex cylinder chamber, with a diameter of 50 cm and height of 50 cm, with a nebulized solution. Saline was delivered instead of OVA in the control group. The polydatin treatment group was pretreated with polydatin (25 mg/kg) prior to OVA stimulation. We euthanized the mice under anesthesia with sodium pentobarbital (30 mg/kg). We cannulated the trachea and rinsed both the airways and lungs in PBS of 1 mL for the BALF collection. We collected the right lungs, which were frozen in liquid nitrogen and stored under -80°C for Western blotting. We preserved the left lungs and fixed them in 4% paraformaldehyde for subsequent immunohistochemical analysis.

Western Blot

Protein from the tumor tissues and cells was extracted and concentrated in a RIPA lysis buffer, which contained a BCA Protein Assay kit (Vigorous Biotechnology Beijing, China) and protease inhibitors (Sigma-Aldrich, St. Louis, USA), respectively. We resolved a total of 20 μ L protein using SDS-PAGE, which was transferred onto a nitrocellulose membrane (Millipore, Madison, USA). The membrane was blocked in 5% nonfat dry milk for 2 h. The membranes were then incubated overnight with primary antibodies at 4°C . Glyceraldehyde 3-phosphate dehydrogenase was utilized as an internal control. We incubated the membrane with horseradish peroxidase-coupled secondary antibodies for 1 h at room temperature after washing.

RNA Extraction and qRT-PCR

Total RNA from lung cells or tissues was extracted using the column method with an RNeasy Mini kit (Qiagen, Valencia, USA) following the standard processes and quantified with the Nanodrop system (Thermo Scientific, Waltham, USA). cDNA was obtained from the extracted RNA by reverse transcription with an RT2 First Strand Kit (Qiagen, New York, USA) according to the manufacturer's instructions. The cDNA was obtained via RT2 SYBRgreen qPCR, which was detected via the $2^{-\Delta\Delta\text{Ct}}$ method. Primers were obtained from Qiagen. U6 and GAPDH were used as the internal controls.

Immunohistochemistry

We fixed lung tissue samples in a 10% formalin solution, after which the samples were embedded into paraffin. The samples were sliced into 5 μ m sections and stained with Masson's trichrome. We observed the sections with an Axiophot light microscope (Zeiss, Oberkochen, Germany) and obtained photographs using a digital camera.

Cell Culture and Treatment

TC-1 cells were obtained from Procell Life Science Co., Ltd. (China), which were cultivated under 37 °C in a CO₂ atmosphere with 5% humidity in Dulbecco's modified Eagle's medium/F12 medium. They were supplied with FBS of 10%, penicillin of 100 U/mL and streptomycin of 100 ng/mL (Invitrogen, Tokyo, Japan). To induce inflammation and airway fibrosis, TC-1 cells (with/without transfection of TGFBR2, circRNA-0000981, or miR-211-5p overexpression vector) were treated with 10 ng/mL TGF- β 1 for 48 h at most.

Transfection

TGFBR2, circRNA-0000981, miR-211-5p overexpression vector, or negative control (NC) were transfected into the cells at an overall concentration of 50 nM using Lipofectamine® 2000 following the standard protocol. Cells were used for subsequent experiments 48 h after transfection.

Bioinformatics Analysis

We predicted the circRNA/miRNA target gene using the *Circular RNA Interactome*. The interaction between miR-211-5p and TGFBR2 was predicted through TargetScanHuman.

Dual Luciferase Reporter Assay

Reporter plasmids were generated by inserting circRNA or the TGFBR2 3'-UTR sequence into the pGL3 vector (Promega, Madison, USA). For the luciferase assay, miR-211-5p mimics and reporter plasmids were co-transfected into 239T cells using Lipofectamine 2000. After a 48-h culture, firefly and Renilla luciferase activity was captured using the Dual Luciferase Reporter Assay System (Promega, Sunnyvale, USA) following the standard procedures.

Statistic Analyses

Data are denoted by the means $\pm$ standard deviation (SD). GraphPad Prism package was used to compare the differences between groups. A *p* value $\leq$ 0.05 indicated a significant difference.

RESULTS

Atractylon Treatment Suppresses OVA-Induced PF and the Relative Expression of EMT Proteins

The immunohistochemistry results revealed that OVA treatment promoted PF, whereas atractylon treatment suppressed OVA induced-PF (Fig. 1a). Moreover, the qRT-PCR results found that atractylon treatment suppressed OVA-induced circRNA-0000981 expression in the lung tissue (Fig. 1b); however, miR-211-5p expression was promoted (Fig. 1c). The Western blot results revealed that atractylon treatment suppressed OVA-induced transforming growth factor beta receptor 2 (TGFBR2) expression (Fig. 1d and e). Increasing reports have found that TGFBR2 is important for the induction of EMT and fibrosis [14, 15]. Atractylon treatment was found to suppress TGFBR2, leading to an inhibitory effect of atractylon on the relative expression of vimentin protein but promoted E-cadherin expression following OVA-induced EMT (Fig. 1f-h). These results also demonstrate that atractylon treatment suppressed the expression of the OVA-induced fibrotropic proteins, α -SMA, collagen I, and collagen III (Fig. 1i-l). Together, these results suggest that atractylon has a protective effect on asthma-mediated PF and EMT related to the regulation of the circRNA-0000981/miR-211-5p/TGFBR2 axis.

CircRNA-0000981 Has an Important Function in TGF- β 1-Induced EMT and Fibrosis-Associated Protein Expression in TC-1 Cells

To identify the interaction between circRNA-0000981, miR-211-5p, and TGFBR2, TC-1 cells stimulated with TGF- β 1 were used as an in vitro model of asthma. The results showed that circRNA-0000981 expression was increased in the TGF- β 1-treated group. However, atractylon treatment suppressed TGF- β 1-induced circRNA-0000981 expression. Following transfection with a miR-211-5p mimic, circRNA-0000981 expression had virtually no effect (Fig. 2a). These data suggest that miR-211-5p functioned downstream of circRNA-0000981. The qRT-PCR results also revealed that TGF- β 1 suppressed miR-211-5p expression, which was recovered following treatment with atractylon. Following transfection with a

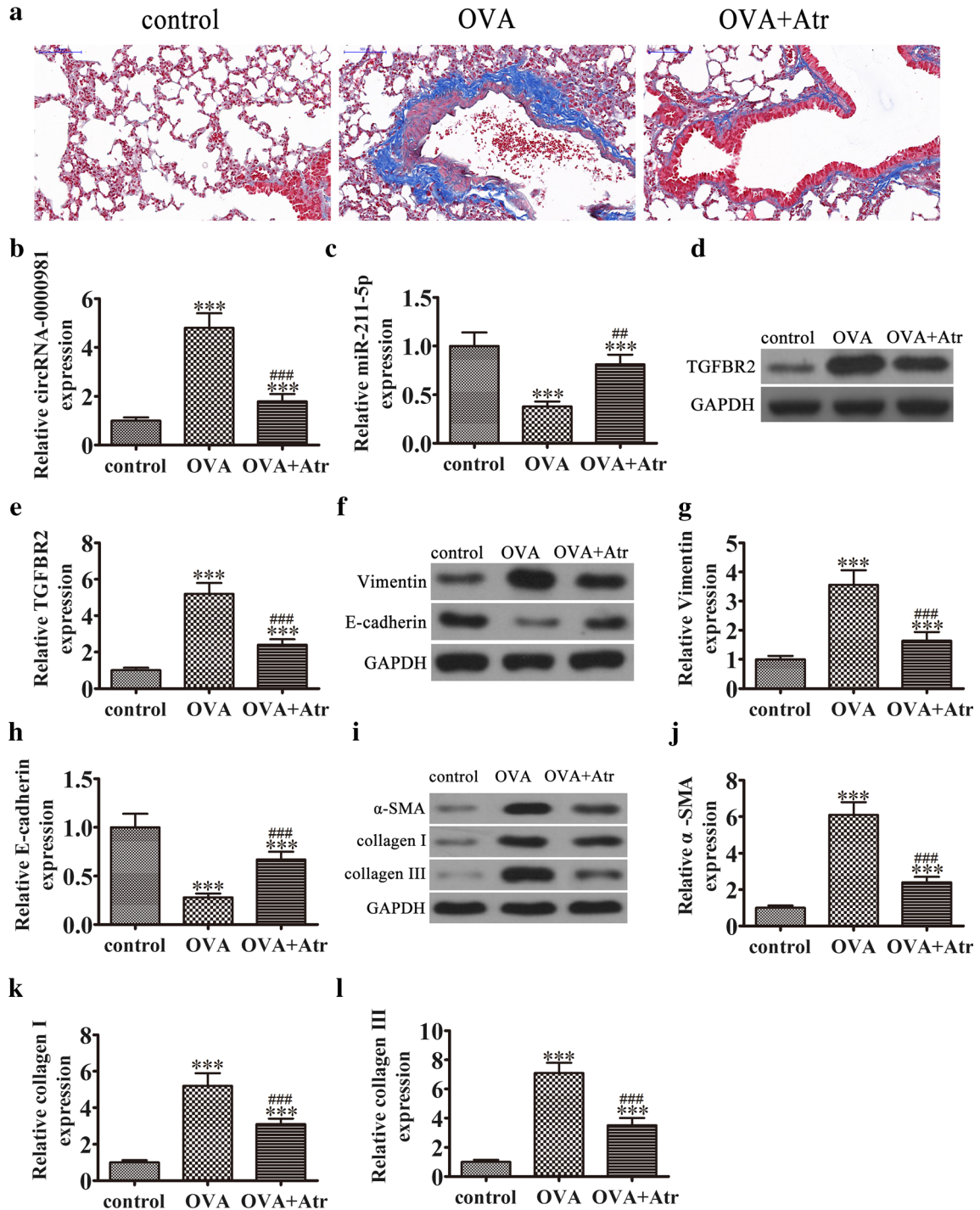


Fig. 1. Atractylon treatment suppressed pulmonary fibrosis in OVA-induced asthma by regulation circRNA-0000981/miR-211-5p/TGFBR2 in asthmatic mice. **a** Immunohistochemistry shows pulmonary fibrosis with Masson's trichrome staining. **b, c** qRt-PCR shows the circRNA-0000981 expression (b) and miR-211-5p expression (c). Values denote the mean $\pm$ S.E.M. *** P < 0.001 versus the control. ## P < 0.01, ### P < 0.001 versus the OVA group. **d, e** Western blot analysis illustrates the TGFBR2 expression level. Values denote the mean $\pm$ S.E.M. *** P < 0.001 versus the control; ### P < 0.001 versus the OVA group. **f-h** Western blot analysis shows the level of E-cadherin and vimentin expression. Values denote the mean $\pm$ S.E.M. *** P < 0.001 versus the control; ### P < 0.001 versus the OVA group. **i-l** Western blot detection shows the expression of α -SMA, collagen I, and collagen III. *** P < 0.001 versus the control; ### P < 0.001 versus the OVA group. Atr, atractylon; OVA, ovalbumin

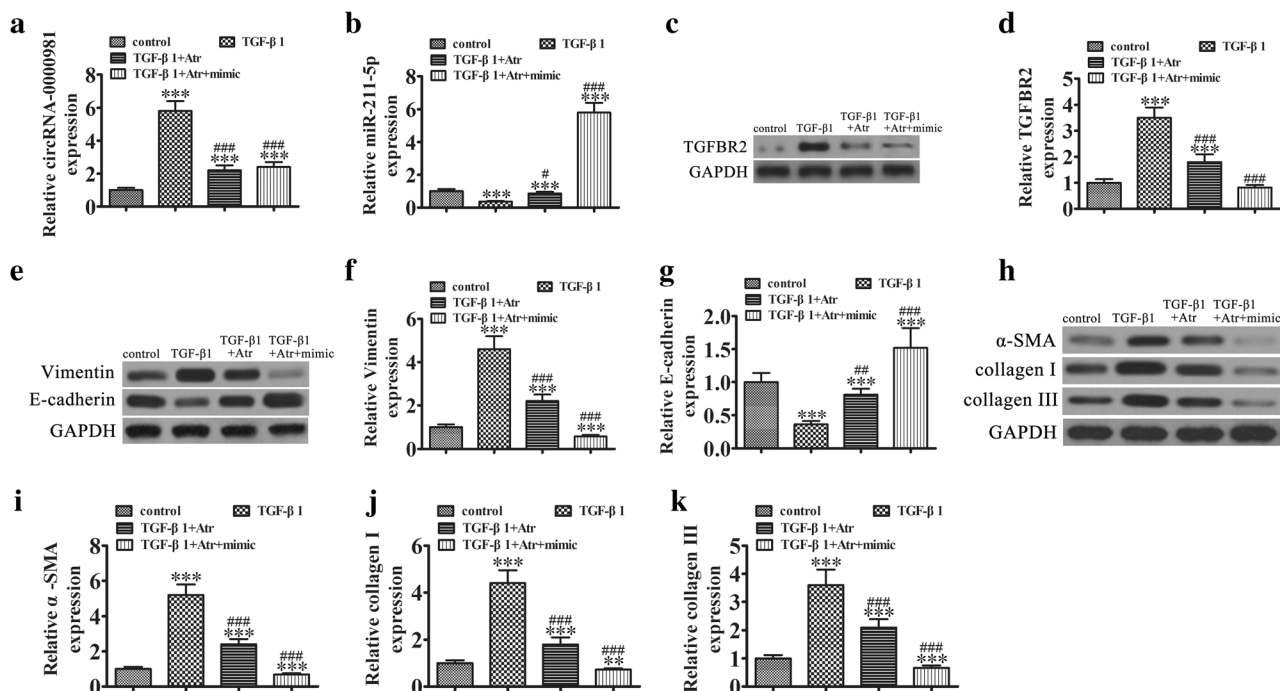


Fig. 2. CircRNA-0000981 functions importantly in TGF- β 1-induced EMT and fibrosis-associated protein expression in TC-1 cells. **a** qRt-PCR detection shows the circRNA-0000981 expression. * P < 0.05, *** P < 0.001 versus the control; #### P < 0.001 versus the atractylon treatment group. **b** qRt-PCR analysis shows the miR-211-5p expression. *** P < 0.001 versus the control; # P < 0.05, #### P < 0.001 versus the TGF- β 1 treatment group. **c, d** Western blot analysis demonstrates the TGFBR2 expression level. Values denote the mean $\pm$ S.E.M. *** P < 0.001 versus the control; #### P < 0.001 versus the TGF- β 1 treatment group. **e-g** Western blot detection shows the E-cadherin and vimentin expression levels. *** P < 0.001 versus the control; ## P < 0.01; #### P < 0.001 versus the TGF- β 1 treatment group. **h-k** Western blot detection shows the α -SMA, collagen I, and collagen III expression levels. ** P < 0.01, *** P < 0.001 versus the control; #### P < 0.001 versus the TGF- β 1 treatment group

miR-211-5p mimic, miR-211-5p expression was significantly increased (Fig. 2b). Western blotting showed that TGF- β 1 promoted TGFBR2 expression, which was suppressed by atractylon treatment. Following transfection with the miR-211-5p mimics, TGFBR2 expression was further suppressed (Fig. 2c and d). The Western blot results revealed that TGF- β 1 promoted the relative expression of EMT-associated vimentin protein but suppressed E-cadherin expression. However, atractylon treatment reversed such TGF- β 1-induced vimentin and E-cadherin expression. Moreover, miR-211-5p promoted the protective effect of atractylon (Fig. 2e, g). The results of the Western blot analyses also found that the expression of the fibrotic proteins, α -SMA, collagen I, and collagen III increased with the induction of TGF- β 1, which was reversed following atractylon treatment. miR-211-5p overexpression further suppressed the expression of α -SMA, collagen I, and collagen III (Fig. 2h-k). These results imply

that the downregulation of circRNA-0000981 expression promoted the level of miR-211-5p.

TGFBR2 Overexpression Reversed miR-211-5p-Induced EMT and Fibrosis-Associated Protein Expression in TC-1 Cells

To identify the interaction between TGFBR2 and miR-211-5p, we transfected TC-1 cells with miR-211-5p mimics or a TGFBR2 overexpression vector either separately or together. The expression miR-211-5p increased substantially following transfection with the miR-211-5p mimics; however, TGFBR2 overexpression had no effect on the expression of miR-211-5p (Fig. 3a). These findings suggest that TGFBR2 is located downstream of miR-211-5p.

The western blot data demonstrated that miR-211-5p overexpression suppressed TGFBR2 expression.

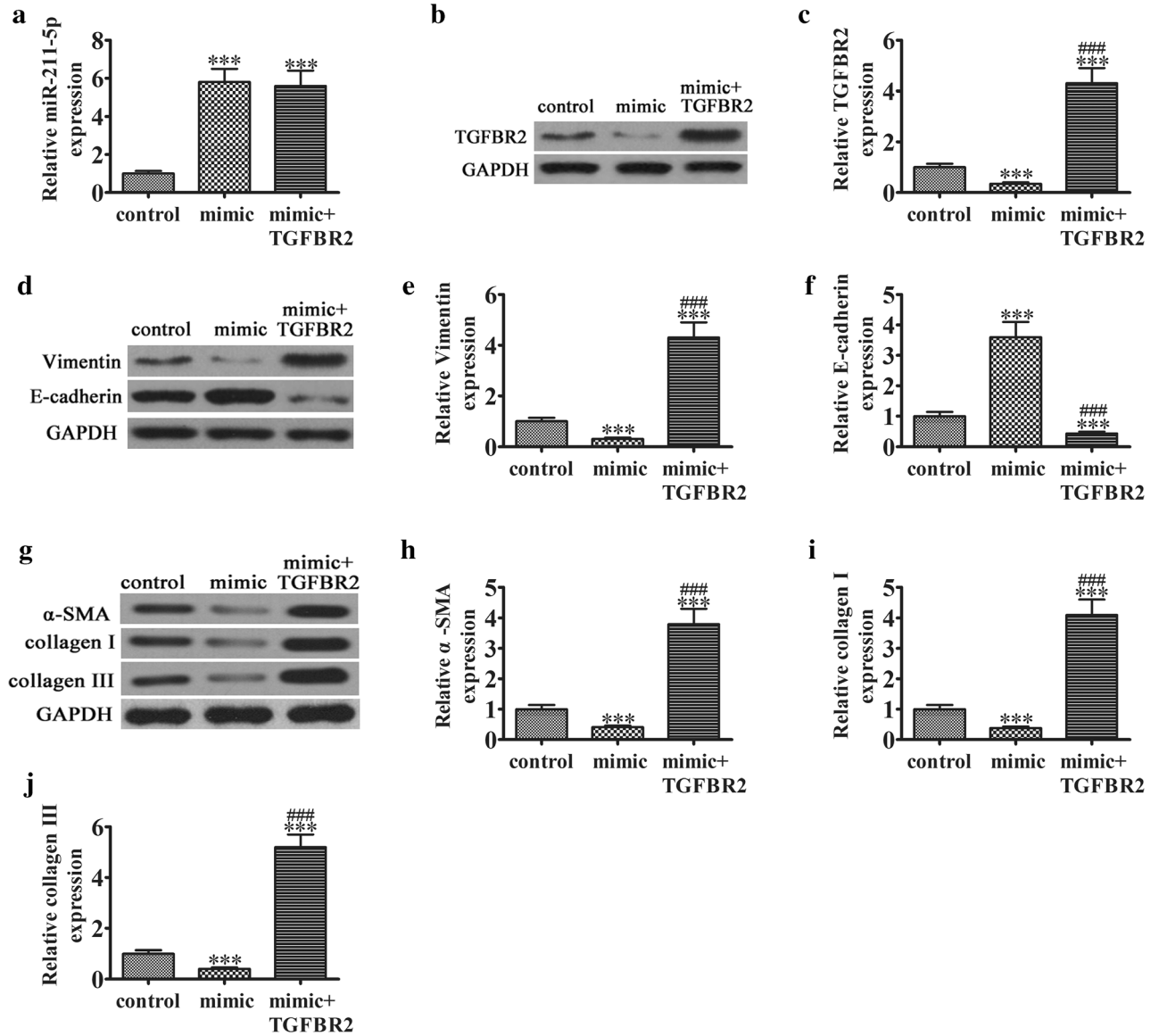


Fig. 3. TGFBR2 overexpression reversed miR-211-5p-induced EMT and fibrosis-associated protein expression in TC-1 cells. **a** qRt-PCR analysis illustrates the miR-211-5p expression. Values denote the mean $\pm$ S.E.M ($n = 3$); *** $P < 0.001$ versus the control. **b, c** Western blot detection shows the TGFBR2 expression level. Values denote the mean $\pm$ S.E.M. *** $P < 0.001$ versus the control; ### $P < 0.001$ versus the mimic group. **d–f** Western blot detection shows the E-cadherin and vimentin expressions. Values denote the mean $\pm$ S.E.M. *** $P < 0.001$ versus the control; ### $P < 0.001$ versus the mimic group. **g–j** Western blot detection shows the α -SMA, collagen I, and collagen III expression levels. Values denote the mean $\pm$ S.E.M. *** $P < 0.001$ versus the control; ### $P < 0.001$ versus the mimic group

Following transfection with the TGFBR2 overexpression vector, TGFBR2 expression significantly increased since the heterologous TGFBR2 has no 3'-UTR, and miR-211-5p cannot recognize and post-transcriptionally silence TGFBR2 expression (Fig. 3b and c). Additionally, miR-

211-5p suppressed the EMT-associated relative expression of vimentin but promoted E-cadherin protein expression, which was reversed by TGFBR2 overexpression (Fig. 3d–f). Thus, miR-211-5p suppressed pulmonary epithelial cell EMT. Expression of the fibrotropic proteins, collagen I, α -

SMA, and collagen III decreased in response to miR-211-5p overexpression but recovered following TGFBR2 overexpression (Fig. 3g–j). Together, these data suggest that miR-211-5p suppressed pulmonary epithelial cell EMT and fibrillation by downregulating TGFBR2.

The Interaction Between circRNA-0000981, miR-211-5p, and TGFBR2

To confirm the correlation between circRNA-0000981, miR-211-5p, and TGFBR2, bioinformatics analyses were performed. The bioinformatics results demonstrated that circRNA-0000981 targeted miR-211-5p. To further verify if miR-211-5p was a potential circRNA-0000981 target, a luciferase reporter assay was performed to verify that miR-211-5p was a circRNA-0000981 downstream binding target (Fig. 4a). Thus, our data revealed that circRNA-0000981 inhibited luciferase activity in wild-type cells but did not impact the activity in the mutated cell lines (Fig. 4b).

To determine whether TGFBR2 was a potential miR-211-5p target, a bioinformatics study was used to verify that miR-211-5p interacted directly with the TGFBR2 3'-UTR and suppressed TGFBR2 expression at the mRNA level (Fig. 4c). A luciferase reporter assay found that miR-211-5p inhibited luciferase activity in the wild-type cells but not in the mutated cell lines (Fig. 4d). These data suggest that silencing circRNA-0000981 inhibited bladder cancer growth and metastasis via targeting the miR-211-5p/TGFBR2 axis.

DISCUSSION

In this study, we found that atractylon treatment suppressed asthma-mediated pulmonary fibrosis and EMT by decreasing circRNA-0000981 and TGFBR2 expression and promoting miR-211-5p. Atractylon is a well-known herb in East Asia, particularly in China. The atractylon rhizome, which is termed “Baizhu” in conventional Chinese medicine, has been applied for a long history to treat a wide range of symptoms, such as spleen hypofunction, diarrhea, abdominal distension, loss of appetite, dizziness, and heart palpitations [16, 17]. Multiple studies have demonstrated that atractylon may have many biological functions, including improving gastrointestinal function, anti-inflammatory, anti-tumor, anti-aging, anti-oxidative, antibacterial, anti-osteoporotic, and neuroprotective activities [18–20]. In this study, we found that atractylon exhibited

anti-fibrotic activity by suppressed circRNA-0000981 expression.

CircRNAs are non-coding RNAs that form closed-loop structures, and their aberrant expression may lead to disease, including cancer [21], cardiovascular disease [22], and Alzheimer's disease [23]. Various studies have been performed to detect the mechanism of fibrosis, such as microvasculature rarefaction, parenchyma injury, fibroblast activation, fibrillar excessive extracellular matrix (ECM) accumulation, and mononuclear infiltrates; however, the underlying mechanism remains poorly understood. The investigation of noncoding RNAs has provided novel insight into the role of circRNA-miRNA-mRNA on disease progression after the development of asthma. In the current study, we found that circRNA-0000981 expression increased in both the OVA-induced asthma and TGF- β 1-treated groups. In addition, the overexpression of circRNA-0000981 suppressed the protective effect of atractylon by promoting the relative expression of EMT and PF-related proteins. PF is a chronic and progressive disease characterized by alveolar epithelial injury and abnormal collagen production caused by the abnormal proliferation of myofibroblasts and fibroblasts, which secrete ECM proteins. Moreover, EMT is an event in which polarized immotile epithelial cells are altered into motile mesenchymal cells. This process is identified by the loss of proteins associated with the polarized epithelial phenotype (e.g., E-cadherin) and an increase in mesenchymal markers (e.g., fibronectin and vimentin). In addition, EMT has been reported to participate in myofibroblast formation [24–27].

We also found that circRNA-0000981 expression increased TGFBR2 levels by sponging miR-211-5. miRNAs are essential biological regulators which function by suppressing their target genes and participate in a substantial number of pathophysiological procedures. Previous studies have found that miR-211-5 participates in the regulation of pulmonary fibrosis [28]. Moreover, our bioinformatics analysis found that TGFBR2 was the target of miR-211-5, which was identified by a luciferase reporter assay. An increasing number of studies have reported that TGFBR2 plays an important role in pulmonary fibrosis by enriching the EMT process [14]. In the present study, the inhibitory effects of miR-211-5 on EMT as well as its target, TGFBR2, were suppressed by circRNA-0000981, which led to PF. However, treatment with atractylon was found to reverse circRNA-0000981-mediated PF by suppressing circRNA-0000981 expression and EMT.

The results of this study highlight the critical function of atractylon in the regulation of asthma pathogenesis.

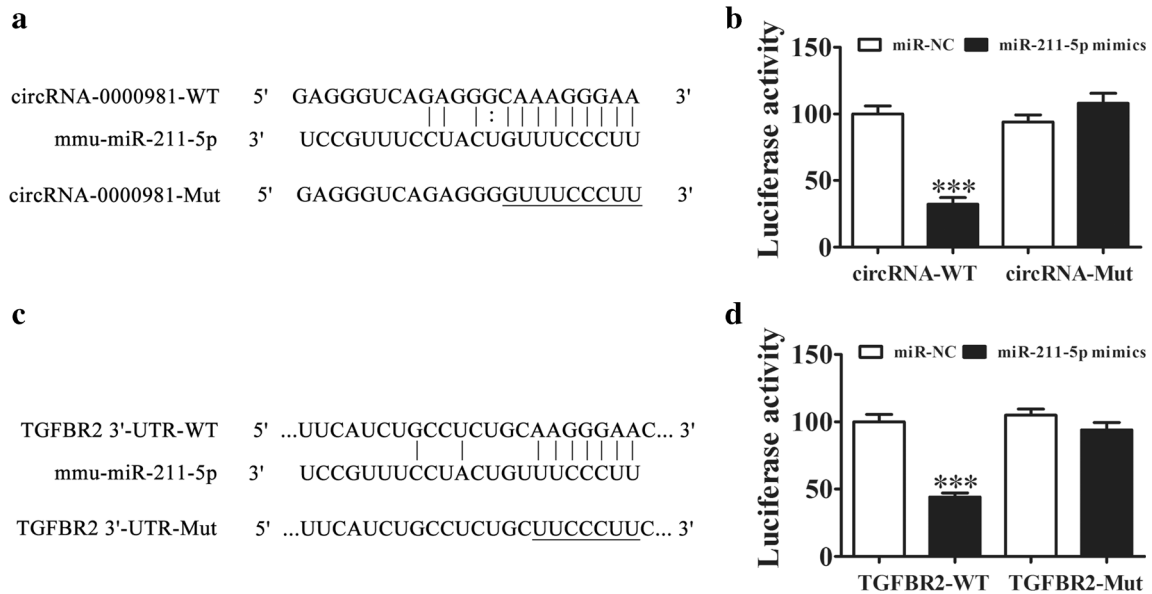


Fig. 4. The interaction between miR-211-5p, circRNA-0000981, and TGFBR2. **a** The predicted miR-211-5p in circRNA-0000981 binding sites. The mutated (Mut) version of circRNA-0000981 is given also. **b** The relative luciferase activity was determined 48 h following transfection with the miR-211-5p mimic/normal control (NC) or with the circRNA-0000981 wild-type/Mut in 293T cells. The data are denoted by the mean $\pm$ SD; *** P < 0.001. **c** The predicted binding sites of miR-211-5p with the TGFBR2 3'-UTR. The mutated version of the 3'-UTR-TGFBR2 is also plotted. **d** Relative luciferase activity was determined 48 h following transfection with the miR-211-5p mimic/normal control or with the 3'-UTR-TGFBR2 wild-type/Mut in 293T cells. Data are denoted by the mean $\pm$ SD; *** P < 0.001

Moreover, these findings suggest that atractylon treatment can attenuate PF by regulating the mmu_circ_0000981/miR-211-5p/TGFBR2 axis in asthma. Hopefully, our research can provide some guidance for the clinical application of atractylodes.

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DECLARATIONS

Conflict of Interest. The authors declare no competing interests.

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