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Original Article

The protective role of Schwann cells in bladder smooth muscle cell fibrosis

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Abstract: Bladder fibrosis is characterized by collagen deposition within bladder walls. Neurogenic lesions are an important contributor to this balder dysfunction. Schwann cells are a kind of glial cell in the peripheral nervous system. However, the role of the cells in bladder fibrosis has received little attention among researchers. Female SD rats were employed in this study to establish a fibrosis model using denervation. Histologically, the fibrosis was evaluated using H&E staining and Masson's staining. CTGF expression was evaluated using immunohistochemistry. Subsequently, the role of Schwann cells in fibrosis was evaluated using a co-culture with bladder smooth cells and exposure to CTGF. After denervation, the bladder fibrosis was observed in a time-dependent manner, and this was accompanied by an increase in CTGF and a decrease in BDNF. After exposure to CTGF, α-SMA, and collagen I and III were significantly increased in the bladder smooth muscle cells. These were significantly inhibited after co-culture with Schwann cells. Furthermore, a significant increase in BDNF was observed in the co-culture. Schwann cells significantly ameliorated the fibrosis of the bladder smooth muscle cells, and this might be associated with the secretion of BDNF.

Keywords: Bladder fibrosis, denervation, bladder smooth cells, Schwann cells, BDNF

Introduction

Bladder fibrosis is a kind of chronic disease in the bladder involving an accumulation of extracellular matrix and decreased parenchymal cells, which can damage to organ and lead to hypofunction, and even threatening health and life. The fibrotic lesion is commonly found in children and was importantly ascribed to neurogenic bladder [1]. However, the potential mechanisms are largely unknown.

Connective tissue growth factor (CTGF), also known as CCN2, is a matricellular protein that is usually expressed at very low levels but which increases significantly in challenged tissues, for example, in bladders under pathological conditions. A few recent studies have shown that the growth factor is usually highly expressed in damaged muscle with fibrotic and necrotic/regenerative lesions [2, 3]. Furthermore, it has been demonstrated that it promotes the proliferation of fibroblasts and the production of ECM using several different cell types, both in vitro and in vivo [4-6]. After employing tests with gain and loss of CTGF function, CTGF has been demonstrated to critically contribute to tissue fibrosis, and it has been shown that CTGF overexpression results in the fibrotic differentiation of smooth cells with a reduction of skeletal muscle strength [5]. On the other hand, decreased levels of CTG show significant protection in muscles against damage, fibrosis, weakness, and resultantly improved cell therapy [7, 8].

Brain-derived neurotrophic factor (BDNF) is a highly conserved growth factor. In addition to being widely expressed in CNS with the critical role in neuronal survival, growth and plasticity, it also is expressed in bladders, the gut, and in other tissues, exerting roles in cell differentiation and chronic inflammation. A recent study has shown that BDNF is involved in lung fibrosis [9]. Schwann cells are a kind of glial cell in the peripheral nervous system responsible for tissue repair resulting from injury to peripheral nervous system. Due to their ability to secrete a

great many neurotrophic factors, the cells are usually used to treat spinal cord injury [10]. A previous study has shown that Schwann cell-like cells differentiated from adipose stem cells reduce fibrosis and promote nerve regeneration in rats [11].

In this study, denervation was conducted on rats to establish a bladder fibrosis model. It was observed that CTGF was significantly increased with the development of fibrosis in the bladder. Furthermore, the data show that Schwann cells significantly ameliorated the fibrosis of bladder smooth muscle cells, and this might be associated with the secretion of BDNF.

Material and methods

Animals and the establishment of bladder fibrosis in vivo

Adult female SD rats weighing 160-200 g were obtained from the Shandong University Laboratory Animal Center and housed in an SPF laboratory under the controlled temperature of 20°C-25°C and a relative humidity of 40%-70% with a 12-hour light-dark cycle. All animals had free access to food and water. All procedures in this study were approved by the ethics committee of the Second Hospital of Shandong University.

The rats were randomly assigned into 4 groups (n = 8/group). One group was given a sham operation and sacrificed 10 days later, while the other groups were denervated and sacrificed at 10, 20, and 30 days later, respectively. The operation was conducted as previously described [12]. In brief, the animals were anaesthetized using sodium pentobarbital (Sigma, 40 mg/kg) and disinfected with an iodine tincture. After making a midline incision at the lower abdomen, we exposed the pelvic plexus and cauterized the major pelvic ganglion in both sides using a stitching pencil. The plexus was exposed, but the ganglion was left intact for the sham-operation rats. A part of the bladder tissue was stored in 4% buffered paraformaldehyde (PFA) until analysis, and the remaining tissue was stored in liquid nitrogen.

Histological and immunohistochemical assays

The bladder tissues fixed in 4% PFA were gradiently dehydrated with ethanol, embedded in paraffin and then cut into 5 μm sections. Subsequently, the sections were dewaxed with dimethylbenzene and rehydrated with ethanol. Some sections were stained using Masson's trichrome staining kit (KeyGen, Nanjin, China) according to the manufacturer's instructions and some with hematoxylin and eosin staining. Some other sections were exposed to 3% hydrogen peroxide for about 5 min to block endogenous peroxidase, followed by being blocked with goat serum. Then all the sections were incubated with the antibody against CTGF (Boster, Wuhan, China) and then with biotinylated anti-rat IgG and HRP-Streptavidin (Boster, Wuhan, China). Finally, the immuno-reaction was visualized by diaminobenzidine and then counterstained with hematoxylin.

Isolation and culture of the bladder smooth cells

The bladder smooth muscle cells (BSMCs) were isolated from the SD rats and were excised as done in a previous study [13]. Briefly, the bladders were harvested in pre-cold PBS containing 100 U/mL penicillin, 100 mg/mL streptomycin and 0.25 mg/mL amphotericin B (Life Technologies, CA, USA). After removal of the mucosa, the bladders were placed into a tissue culture dish with pre-cold PBS. The serosal and mucosal sides received a gentle scrape by a cotton swab to completely remove the urothelial and mucosal cells. Subsequently, the bladders were minced into fragments of 1-3 mm^3. After being placed into a six-well tissue culture plate, the fragments were cultivated with DMEM containing 10% FBS and the abovementioned antibiotics for 2 h at 37°C. After 3 days, the medium was refreshed every other day until confluence. The cells were trypsinized and collected for the following examinations.

Isolation of Schwann cells and co-culture with BSMCs

Under aseptic conditions, Schwann cells were isolated from the sciatic nerves of the neonatal SD rats (1-2 days old) and then immersed in Hank's balanced salt solution with 1% (v/v) penicillin/streptomycin. On ice, the blood vessels and connective tissue were removed under a microscope. The sciatic nerves were cut into fragments of 1-mm^3 and then dissociated with 0.15% collagenase (Sigma, UK) and 0.25% trypsin (Sigma, UK) for about 30 min at 37°C.

Then the medium with 15% FBS was added to neutralize the solution. After centrifugation at 1000 g for 5 min and a subsequent resuspension, the cells were maintained in DMEM with 10% FBS and cultured at 37°C in 5% CO_2.

Transwell culture dishes with a polycarbonate membrane of 1.0 μm pore size (Millipore, USA) were used to co-culture BSMCs and Schwann cells. In brief, BSMCs ($6\times10^4/cm^2$) were cultured in the well, and SCs (3×10^4 cells/cm^2) were cultured on the permeable membrane support. Both cells were maintained in DMEM with 10% FBS and exposed to 15 ng/mL CTGF.

Cell treatment

The cells (1×10^5 cells/well) were plated in 6-well plates with a 3 mL medium. After 24 h incubation, the cells were exposed to 15 ng/mL CTGF to establish bladder fibrosis *in vitro*. After 24 h, the cells were fixed with 4% PFA and received examinations.

α-SMA staining

The fixed cells were blocked with 5% horse serum, and then they were incubated overnight at 4°C with anti-SMA (Sigma Chemical) and then with Alexa Fluor 488-conjugated secondary antibody (Molecular Probes) for 1 hour. The staining was observed under fluorescence microscopy.

Western blot analysis

The cultured BSMCs were lysed using a RIPA buffer (Beyotime, Haimen, China), and the protein concentrations were determined with a BCA protein assay kit (Beyotime, Haimen, China). After denaturing at 95°C for 5 min, 20 μg protein of each group was subjected to electrophoresis by 10% sodium dodecyl sulfate-polyacrylamide gel. Following their transfer to a PVDF membrane, the blots were blocked with 5% skim milk and were incubated with the primary antibodies against Collagen I and III (Boster, Wuhan, China) at 4°C overnight. After being washed with TBST, the blots were incubated with horseradish a peroxidase-conjugated secondary antibody (Beyotime, Haimen, China) at 37°C for 30 min, and then they were visualized using an enhanced chemiluminescence (ECL) detection system. GAPDH was used as the internal reference.

ELISA

Using an ELISA kit (Elabscience, Wuhan, China), BDNF was quantified in the collected bladders and culture mediums following the manufacturer's instructions. Three replicates were performed.

Senescence-associated beta-galactosidase cytochemical assay

At the end of the co-culture, SCs were fixed with 4% PFA and then washed with PBS. Subsequently, a senescence-associated beta-galactosidase (SA-β-gal) staining solution was added, and the cells were incubated with a staining solution at 37°C for about 8 h. After being washed twice with PBS, the SA-β-gal-positive cells were observed under a BX51 lighted microscope (Olympus, Japan).

Statistical analysis

Data in this study are expressed as the means ± SDs. Unpaired *t* tests were used for the statistical analysis and a statistically significant difference was considered when $P<0.05$. All experiments were repeated three times.

Results

Significantly decreased bladder BDNF in bladder fibrosis rats

To observe the expressions of BDNF in bladder tissues with fibrosis, we first performed denervation to establish a tissue fibrosis model. H&E staining showed that the smooth muscle significantly decreased in a time-dependent manner (**Figure 1A**). This was consistent with Masson's staining which exhibited a significant increase in fibrosis (**Figure 1B**). These results indicated that bladder fibrosis was successfully established in the rats.

Subsequently, CTGF was examined by IHC. As shown in **Figure 1C**, CTGF expressions were significantly increased in the model tissues, indicating it might play an important role in fibrogenesis. By ELISA, BDNF was examined and showed a significant and time-dependent decrease in the rats with denervation (**Figure 2**), indicating that BDNF might function as protector of bladder tissues from fibrosis.

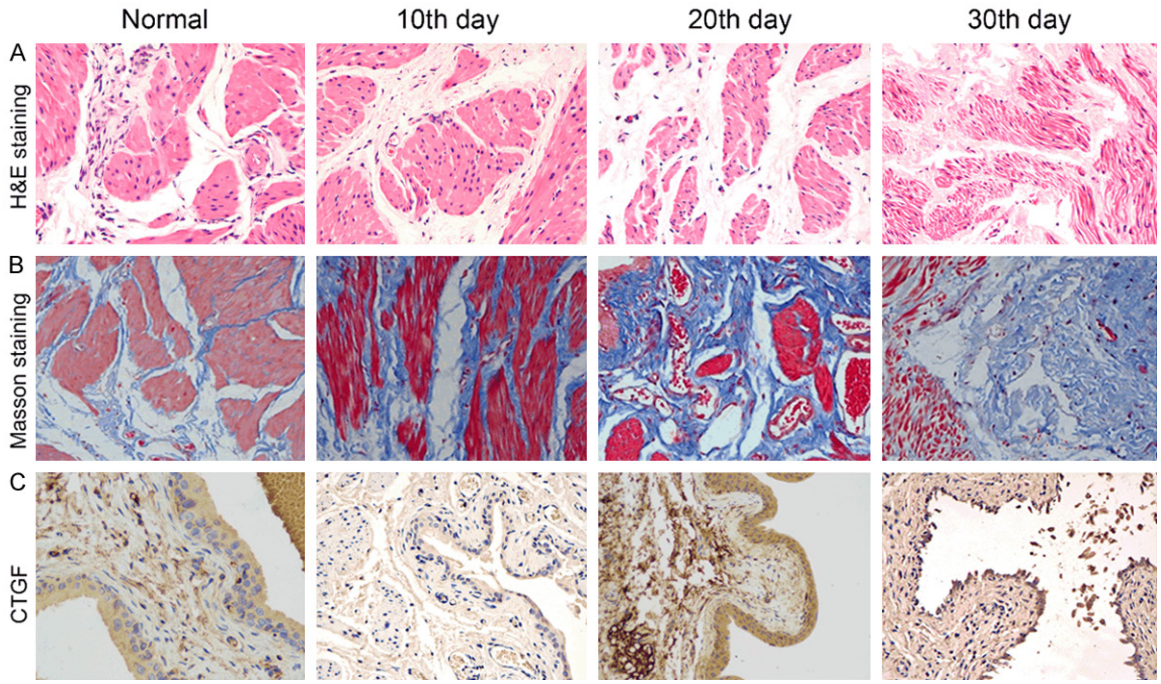

Figure 1. The accumulation of fibrosis in bladder tissues. The bladders given a sham or denervation operation were sampled and then received hematoxylin and eosin (A) or Masson's (B) staining. Additionally, the expressions of CTGF in the tissues were determined using immunohistochemistry (C). Magnification = 400×.

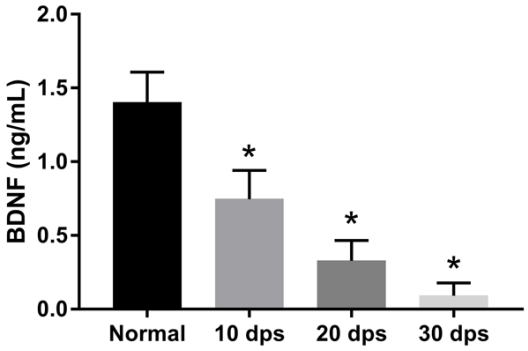

Figure 2. Decreased BDNF expression in fibrotic bladder tissues. After denervation, a decrease in BDNF in bladder tissues was observed in a time-dependent manner. *P<0.05 vs. Normal group. BDNF, Brain-derived neurotrophic factor.

CTGF induced fibrosis in BSMCs

To confirm the effect of CTGF on fibrogenesis, the primary BSMCs were isolated and then cultured with CTGF. As shown in **Figure 3A**, α-SMA was significantly increased by treatment with CTGF. Western blot showed that collagen I and III were significantly increased (**Figure 3B**). These results indicated that CTGF might play an important role in fibrosis generation in BSMCs.

Schwann cells significantly attenuated fibrosis in BSMCs

Co-culture was employed to evaluate the effect of Schwann cells on BSC fibrosis induced by CGGF. As shown in **Figure 4A**, Schwann cells significantly decreased the expression of α-SMA in BSMCs. Additionally, collagen I and III in BSMCs were also significantly decreased after co-culture with Schwann cells (**Figure 4B**). These results indicated that Schwann cells can significantly attenuate BSC fibrosis induced by CTGF and might exert a protective function in bladder fibrosis. A further ELISA assay showed that BDNF in the medium was significantly increased in the co-culture system, as compared with the culture of BSC alone and exposed to either CTGF or a vehicle, indicating that BDNF might play a mediatory role in the attenuative effect by Schwann cells (**Figure 4C**). SA-β-gal staining showed that the positive densities were comparable among the groups, indicating senescence did not occur in the Schwann cells (**Figure 5**).

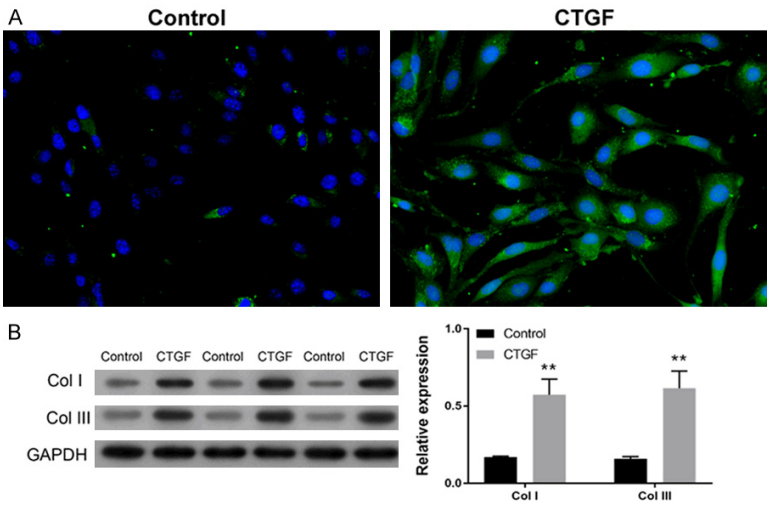

Figure 3. Fibrotic alterations in BSMCs exposed to CTGF. After exposure to CTGF, increased expression of α-SMA was observed by immunofluorescence staining (A), and Col I and III were observed by western blot (B). $^{**}P<0.01$ vs. other group. CTGF, connective tissue growth factor (CTGF); BSMCs, bladder smooth muscle cells; Col I, collagen I; Col III, collagen III.

Discussion

The bladder fibrosis was characterized by a thickening of the BSMCs and the epithelial layers and increased ECM proteins. In the present study, the bladder fibrosis was established by denervation *in vivo* and co-culture *in vitro* with BSMCs and Schwann cells. And we, for the first time, observed that CTGF might play an important role in denervation-induced bladder fibrosis, and Schwann cells might function as an important protector from BSC fibrosis. Furthermore, our data indicated that BDNF might play an important mediatory role.

After the denervation was carried out in this study, CTGF was significantly increased in a positive association with bladder fibrosis. Along with the pro-fibrogenic function of the growth factor, it was strongly suggested to play an important role in denervation-induced bladder fibrosis. As an important member of peripheral nerves, Schwann cells critically contribute to shaping peripheral nerves, and the tissue repair resulted from the peripheral nervous injury. In this study, Schwann cells significantly inhibited CTGF-induced fibrosis of BSMCs. This was reported to be mostly attributed to the ability to secrete various neurotrophic factors, such as BDNF [10, 14]. Consistently, it was observed in the present study that Schwann cells significantly increase the medium levels of BDNF after being exposed to CTGF. Although there are few studies on the effect of CTGF on Schwann cells, BDNF has been shown to act on the cells of the central system and have been shown to release CTGF in response to stimuli [15]. Therefore, we hypothesized that the growth factor might activate Schwann cells both in a direct way or indirect way, leading to the marked release of BDNF.

In addition to the initial recognition in the nervous system, BDNF has been confirmed in various tissues, including bladder, lung and liver tissue [16-19]. Consistently, BDNF was detected in the bladder tissues in the present study. Furthermore, it was decreased with the severity of bladder fibrosis. Additionally, the in vitro results showed the increased medium BDNF coincided with the decreased degeneration of BSMCs. These findings indicated that BDNF might play an important protective role in fibrotic lesions in bladder. The factor has attracted attention as a potential biomarker of lower urinary tract symptoms, which often results from bladder outlet obstruction and is associated with bladder fibrosis [20]. Furthermore, it has been proven that BDNF is more of a cytokine associated with bladder function and bladder remodeling [21]. As a multifunctional growth factor, BDNF exerts pro-activity in BSMCs and has a functional role in the BSC phenotype. All these indicated the important protective role of BDNF in BSMCs by directly regulating the activity and phenotype of the cells. Additionally, BDNF was demonstrated to promote angiogenesis, maintain survival of endothelial cells and smooth cells, as well as mediate inflammation, all of which were shown to protect the corresponding tissues, including heart and nervous system tissues.

It has been thought that fibrosis is common in chronic diseases of the lung, liver, heart, kidney, and bladder [22]. Therefore, bladder fibrosis is believed to follow the development of fibrogenesis similar to its development in other organs [23]. At the initiation of fibrosis, activated fibroblasts firstly translocate to injured tis-

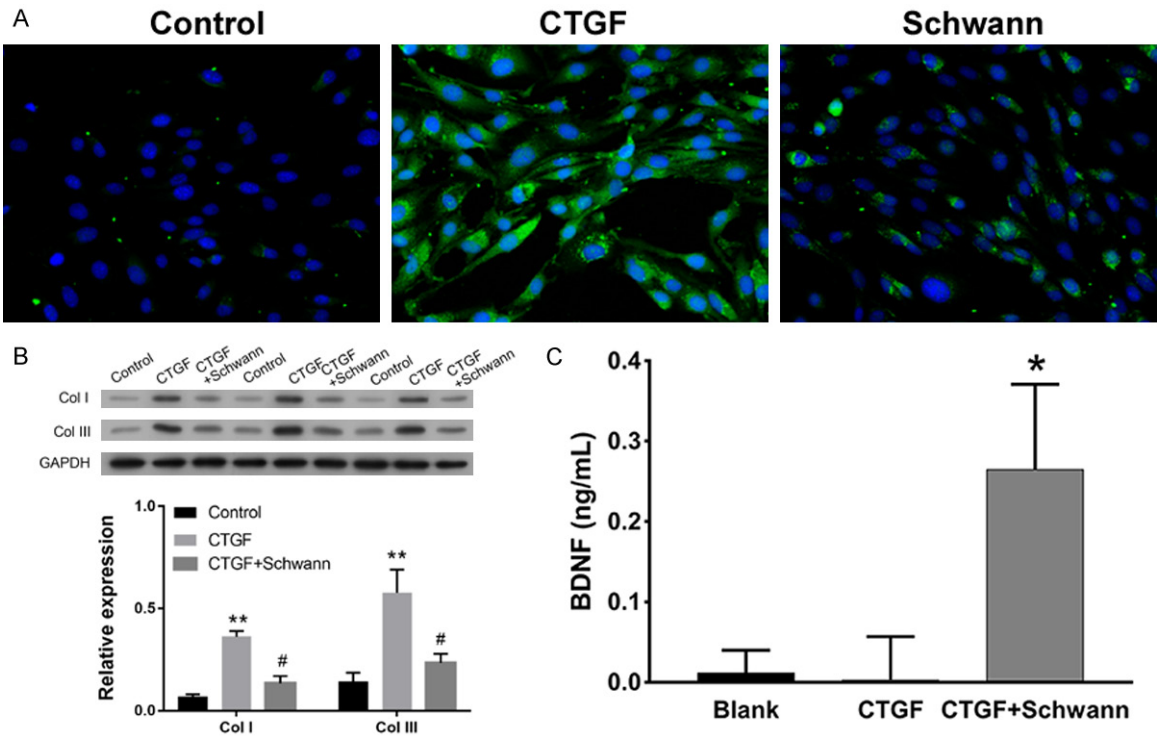

Figure 4. Co-culture with Schwann cell-inhibited fibrotic alterations in BSMCs. In the co-culture system treated with CTGF, decreased α-SMA was observed using immunofluorescence staining (A), decreased expressions of Col I and III were determined using a western blot assay (B), and increased levels of BDNF were observed using an ELISA assay (C), when compared with the culture of BMSCs alone. *P<0.05, **P<0.01 vs. control group; #P<0.05 vs. CTGF group.

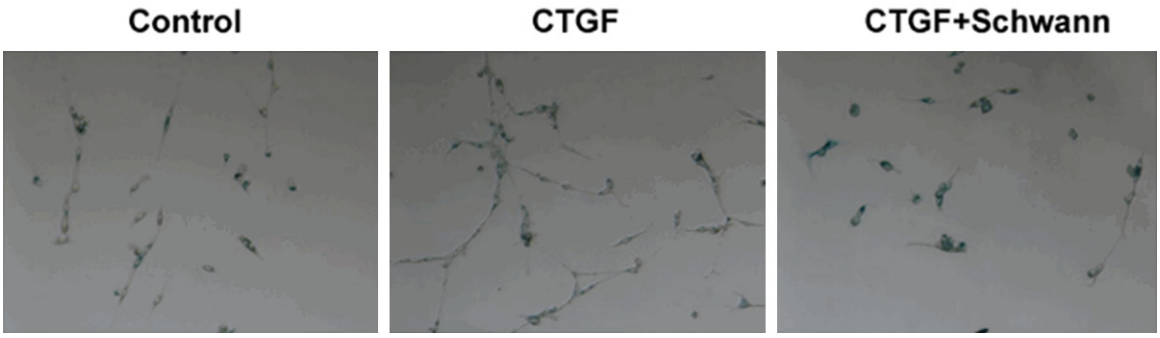

Figure 5. The senescence of the Schwann cells was determined using SA-β-gal staining.

sue to repair wounds. As ECM accumulates, pathological fibrosis occurs, eventually leading to organ malfunction [24, 25]. Similar to BSMCs, BDNF increases aveoliar smooth cell contractility and hyperactivity, indicating that the role of BDNF in pulmonary fibrosis maybe consistent with its role in bladder fibrosis. Unexpectedly, it has been reported that exogenous BDNF significantly increases collagen-1, collagen-3, and fibronectin (intracellular) expression in aveoliar smooth cell, in addition to increased expressions of MMP-2 [16]. Furthermore, this study also indicated that BDNF might act as a contributor to airway fibrosis, which has a prerequisite of inflammation. This discrepancy might result from the etiology.

Conclusion

The attenuative effect by Schwann cells on BSC fibrosis was observed in this study, and this might be associated with BDNF.

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Disclosure of conflict of interest

None.

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