

Anti-fibrotic effect of *Sedum sarmentosum* Bunge extract in kidneys via the hedgehog signaling pathway

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Received April 8, 2016; Accepted March 10, 2017

DOI: 10.3892/mmr.2017.6628

Abstract. *Sedum sarmentosum* Bunge (SSBE) is a perennial plant widely distributed in Asian countries, and its extract is traditionally used for the treatment of certain inflammatory diseases. Our previous studies demonstrated that SSBE has marked renal anti-fibrotic effects. However, the underlying molecular mechanisms remain to be fully elucidated. The present study identified that SSBE exerts its inhibitory effect on the myofibroblast phenotype and renal fibrosis via the hedgehog signaling pathway *in vivo* and *in vitro*. In rats with unilateral ureteral obstruction (UO), SSBE administration reduced kidney injury and alleviated interstitial fibrosis by decreasing the levels of transforming growth factor (TGF)- β 1 and its receptor, and inhibiting excessive accumulation of extracellular matrix (ECM) components, including type I and III collagens. In addition, SSBE suppressed the expression of proliferating cell nuclear antigen, and this anti-proliferative activity was associated with downregulation of hedgehog signaling activity in SSBE-treated UO kidneys. In cultured renal tubular epithelial cells (RTECs), recombinant TGF- β 1 activated hedgehog signaling, and resulted in induction of the myofibroblast phenotype. SSBE treatment inhibited the activation of hedgehog signaling and partially reversed the fibrotic phenotype in TGF- β 1-treated RTECs. Similarly, aristolochic acid-mediated upregulated activity of hedgehog signaling was reduced by SSBE treatment, and thereby led to the abolishment of excessive ECM accumulation. Therefore, these findings suggested that SSBE attenuates the myofibroblast phenotype and renal fibrosis via suppressing the hedgehog signaling pathway, and may facilitate the development of treatments for kidney fibrosis.

Introduction

Chronic kidney disease has become a major global public health problem, and places great burden on affected individuals, families and societies. Despite the enormity of this problem, current therapeutic options for chronic kidney disease in the clinical setting are often ineffective (1). Interstitial fibrosis is considered as the ultimate common pathway for chronic kidney diseases (2). However, the molecular mechanisms underlying interstitial fibrosis in kidney tissues are not fully understood.

Previous studies have demonstrated that activated hedgehog signaling promotes renal fibrogenesis (3,4). Hedgehog-mediated fibrotic alterations are associated with enhanced expression of transforming growth factor (TGF)- β 1 (3,5,6), and promotes myofibroblast formation of renal tubular epithelial cells (RTECs), endothelial cells, pericytes and activated fibroblasts. As a result, excessive accumulation of extracellular matrix (ECM) components in kidney tissues induces interstitial fibrogenesis. Thus, it is of significance to search for effective therapies to suppress the hedgehog signaling-mediated fibrotic phenotype, and renal fibrosis.

Our previous studies demonstrated that the extract of *Sedum sarmentosum* Bunge (SSBE), a perennial plant that is widely distributed on the mountain slopes of Asian countries and contains multiple active flavonoids (such as quercetin, isorhamnetin and kaempferide) (7-9), has marked renal anti-fibrotic effects (10,11). In aristolochic acid (AA)-treated RTECs, SSBE induces cellular apoptosis and inhibits proliferation. These anti-proliferative effects of SSBE impede myofibroblast formation, and may occur as a result of abnormal proliferation of RTECs via epithelial-to-mesenchymal transition (EMT). Over-activation of hedgehog signaling is responsible for abnormal proliferation by regulating components of the cell cycle, such as c-Myc and cyclin D1 (12,13). Thus, it was hypothesized that SSBE may have an inhibitory effect on hedgehog signaling. To test this hypothesis, the present study examined the effects of SSBE on renal fibrosis induced by ureteral obstruction *in vivo*, and production of ECM components induced by AA or TGF- β 1 *in vitro*. Furthermore, the activity of the hedgehog signaling pathway was evaluated.

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Key words: *Sedum sarmentosum* Bunge, hedgehog signaling, aristolochic acid, transforming growth factor- β 1, renal fibrosis

Materials and methods

Animal model and tissue preparation. Male Sprague-Dawley rats (weight, 180–200 g; age, 6–8 weeks; $n=32$) were purchased from the Experimental Animal Center of Wenzhou Medical University (Wenzhou, China). Rats were housed under a controlled temperature (22–25°C), humidity (40–60%) and light environment (12-h dark/light), and fed with standard rat chow (10–15 g twice a day) and water (20–45 ml a day), and this access was controlled, except for one day of fasting prior to the operation. The weight-matched rats were randomly assigned to one of four groups: Sham-operated, treated with vehicle (saline, $n=8$) or SSBE (100 mg/kg/day, $n=8$), and unilateral ureteral obstruction (UO) treated with vehicle ($n=8$) or SSBE (100 mg/kg/day, $n=8$). UO surgery was performed as previously described (11). All rats were sacrificed by cervical dislocation and were anesthetized by 0.2% pentobarbital sodium (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). Kidneys were excised at day 8 for rats in the UO SSBE and vehicle control groups as previously described (11). SSBE (cat. no. 20101017; Xuancheng Baicao Plant Industry and Trade Co., Ltd., Anhui, China) was extracted according to the standard protocol (10). The animal study protocols were approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University.

Renal histology and immunohistochemistry. The paraffin-embedded kidney sections were stained using standard histology procedures as previously described (11), including hematoxylin and eosin (H&E) and Masson's trichrome staining (both from Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China). Immunohistochemical analysis was performed on 4- μ m-thick kidney sections using an automatic slicing machine (YD-335; Wuxiang Instrument, Shanghai, China) that had been dewaxed with xylene and rehydrated using sequential ethanol (100, 95, 85 and 75%) and distilled water. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 30 min. Antigen retrieval was performed by heating the sections in 0.1% sodium citrate buffer (pH 6.0). Immunohistochemical analysis was performed using anti-TGF- β 1 (dilution 1:800, cat. no. bs0103R; BIOSS, Beijing, China), anti-type III collagen (Col3 α 1; dilution 1:800, cat. no. bs-0549R; BIOSS) and anti-proliferating cell nuclear antigen (PCNA; dilution 1:1,000, cat. no. sc-9857; Santa Cruz Biotechnology, Dallas, TX, USA) primary antibodies at 4°C overnight and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (dilution 1:10,000, cat. no. P0211; Beyotime Institute of Biotechnology) at 37°C for 30 min. The integrated optical density was measured using Image-Pro Plus version 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). All samples were semi-quantitatively or quantitatively assessed by two blind independent investigators.

Cell culture and drug treatment. The NRK-52E renal epithelial cell line was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China), and was maintained in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 5% fetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen). NRK-52E cells were

seeded into 6-well culture plates at a density of 3×10^5 cell/well to confluence in complete medium containing 5% FBS for 24 h, and then changed to serum-free medium for 24 h before treatment with 5 ng/ml TGF- β 1 (cat. no. 0312209-1; PeproTech, Inc., Rocky Hill, NJ, USA), 10 μ g/ml AA (cat. no. A5512; Sigma-Aldrich) or 10–1,000 μ g/ml SSBE.

Immunofluorescence staining. NRK-52E cells were cultured with TGF- β 1, AA, and/or SSBE in 6-well plates at a seeding density of 3×10^5 cells/well containing glass slides. Cells were washed with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde (Sigma-Aldrich) at 4°C for 30 min. Following permeabilization with 0.1% Triton X-100 for 10 min, specimens were washed with PBS, and the substrate was blocked with 10% FBS to eliminate nonspecific fluorescence. Immunofluorescence staining was performed using anti-Col3 α 1 (dilution 1:200), anti-E-cadherin (cat. no. ab53033, dilution 1:400; Abcam, Cambridge, MA, USA), and anti- α -smooth muscle actin (α -SMA; dilution 1:400; cat. no. sc-32251), anti-protein patched homolog 1 (Pth1; dilution 1:400; cat. no. sc-9016), anti-smoothed (Smo; dilution 1:400; cat. no. sc-13943) and anti-Gli family zinc finger 1 (Gli1; dilution 1:100; sc-6153), purchased from Santa Cruz Biotechnology, primary antibodies at 4°C overnight. Following washing with PBS three times, the cell preparations were incubated with fluorescein isothiocyanate (green)/tetramethylrhodamine-(red) labeled secondary antibodies (dilution 1:2,000; Sigma-Aldrich) for 1 h at room temperature. Following washing with PBS, cell preparations were placed in acacia and covered with a slide. Immunofluorescence studies were semi-quantitatively or quantitatively assessed by two blind independent investigators.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from NRK-52E cells or kidney tissues using TRIzol® reagent (Invitrogen). Reverse transcription into cDNA templates were performed using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). qPCR was performed using a SYBR-Green Real-Time PCR Master Mix Plus (Toyobo). Quality was analyzed on agarose gels, and quantities were measured using Varioskan Flash (Thermo Fisher). Sequence-specific primers of α -SMA, tight junction protein 1 (ZO-1), type I collagen (Col1 α 1), Col3 α 1, sonic hedgehog (Shh), Pth1, Smo, Gli1, TGF- β 1 and TGF- β 1 receptor (TGF β 1R), all listed in Table I, were synthesized by Invitrogen; Thermo Fisher Scientific, Inc., and β -actin served as an endogenous reference gene. Samples were analyzed in triplicate. The melting curve was examined to verify that a single product was amplified. For quantitative analysis, all samples were analyzed using the $2^{-\Delta\Delta C_q}$ value method (14). For semi-quantitative analysis, all samples were analyzed using gel electrophoresis.

Western blot analysis. Whole proteins from NRK-52E cells were collected using RIPA lysis buffer (Beyotime Institute of Biotechnology) by centrifugation at 12,900 \times g for 10 min, and protein concentrations were determined using a Bicinchoninic Acid protein assay kit (Beyotime). Whole proteins (30 μ g) from each sample were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Beijing Solarbio Science & Technology, Beijing, China). Following blocking with 5% skimmed milk at 37°C for 1.5 h, membranes were

Table I. Primers for reverse transcription-quantitative polymerase chain reaction analysis.

Gene	GenBank accession no.	Forward primer (5'→3')	Reverse primer (5'→3')
Col1α1	NM_053304.1	GATCCTGCCGATGTCGCTAT	GGAGGTCTTGGTGGTTTTGTATTC
Col3α1	NM_032085.1	AAGGCTGAAGGAAATAG	AATGTCATAGGGTTCGATA
Ptch1	NM_053566.1	TCCAGCCGACCCAGATTG	ACATAGTCGTAGCCCCTGAAGTG
Shh	NM_017221	ACAAGAAACTCCGAACGATT	ACAAGAAACTCCGAACGATT
Smo	NM_012807.1	TGTGGCTCAGGTAGATGG	GGTGGTTGCTCTTGATGG
Gli1	XM_006241443.2	CCTCGTGGCTTTCATCAACTCT	GAAGCATCATTGAACCCTGAGTAGA
ZO-1	NM_001106266.1	GGCATCCACGAAACCACCT	CCGCCGATCCAGACAGAAT
α-SMA	NM_031004.2	AACAGAGCCGAGCAGTTAGCC	CAACATCAGCAATCGGTCCA
TGF-β1	NM_021578.2	AGGCGGTGCTCGCTTTGT	GATTGCGTTGTTGCGGTCC
TGF-β1R	NM_012775.2	TGATCCATCCGTTGAAGAAA	CTAGCTGCTCCATTGGCATA
β-actin	NM_031144.2	CCCATCTATGAGGGTTACGC	TTTAATGTACGCACGATTTC

TGF-β1, transforming growth factor-β1; Smo, smoothened; Ptch1, protein patched homolog 1; Col1α1, type I collagen; Col3α1, type III collagen; α-SMA, α-smooth muscle actin; Shh, sonic hedgehog; Gli1, Gli family zinc finger 1; R, receptor; ZO-1, tight junction protein 1.

incubated with anti-Col3α1 (dilution 1:1,000), anti-α-SMA (dilution 1:1,000) and anti-Smo (dilution 1:1,000) primary antibodies at 4°C overnight, and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (dilution 1:5,000; Beyotime Institute of Biotechnology) at 37°C for 2 h. Bound antibodies were visualized using chemiluminescence detection (ECL, cat. no. 32109; Thermo Fisher Scientific, Inc.) on autoradiographic film. Quantification was performed by measuring the intensity of signals using Image-Pro Plus version 6.0 software (Media Cybernetics), and normalized to that for the anti-GAPDH antibody (dilution 1:2,000; cat. no. AP0063; Bioworld Technology).

Statistical analysis. All results are presented as mean ± standard error. Statistical analyses were performed using a Statistical Package for Social Sciences version 16.0 software (SPSS, Inc., Chicago, IL, USA). Student's t-test was used to analyze differences between the two groups, and one-way analysis of variance followed by least significant difference post hoc test was used for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

SSBE reduces TGF-β1 expression and alleviates interstitial fibrosis in UUO kidneys. Evidence from H&E staining revealed marked tubular dilation and atrophy associated with interstitial fibrosis in the obstructed kidney tissues (Fig. 1A). The total collagen deposition determined by Masson trichrome staining was more severe as obstructive time progressed (Fig. 1A). SSBE administration significantly alleviated renal tubular injury and reduced total collagen deposition (Fig. 1A). These findings suggested that SSBE alleviated UUO-induced interstitial fibrosis in rats.

Compared with those in the sham-operated group, the mRNA expression levels of Col3α1 (Fig. 1B), and the protein expression levels of Col1α1 and Col3α1 (Fig. 1C) in UUO kidneys were significantly increased. These results supported that UUO induced excessive ECM deposition and interstitial

fibrosis in kidney tissues. The fibrotic alterations in UUO kidneys were associated with enhanced gene (Fig. 1B) and protein (Fig. 1C) expression of the profibrotic factor TGF-β1, and mRNA expression of its receptor TGF-β1R (Fig. 1B). However, the upregulated expression of TGF-β1, TGF-β1R and ECM components were inhibited by the treatment of SSBE. Furthermore, SSBE suppressed cellular proliferation in the tubules and interstitium by reducing the numbers of PCNA-positive cells (Fig. 1D). Therefore, the inhibitory effect of SSBE on cellular proliferation indirectly regulates the tubular epithelial cell phenotype and myofibroblast accumulation, resulting in the reduction of interstitial fibrogenesis.

SSBE inhibits the activation of hedgehog signaling in UUO kidneys. UUO has been demonstrated to induce cell proliferation in kidney tissues, which may occur via a feedback model, and is accompanied with activation of proliferation-associated signaling, including the hedgehog signaling pathway (15). Therefore, the present study examined the gene and protein expression levels of key molecules involved in the hedgehog signaling pathway, in the obstructed kidney. As presented in Fig. 1E, UUO induced the synthesis and secretion of Smo, and inhibited the expression of Ptch1, a hedgehog inhibitor by targeting Smo. In addition, upregulated mRNA expression levels of Gli1 and downregulated expression levels of Ptch1 were observed in UUO kidneys (Fig. 1F). These findings suggested that UUO induced the activation of hedgehog signaling. Previous studies have demonstrated that in UUO rats, hedgehog signaling is activated by a paracrine signaling loop and mediates epithelial-mesenchymal communication and promotes renal fibrosis (3,4). Blockade of hedgehog signaling may alleviate the extent of fibrosis (3,16). In the present study, the activity of hedgehog signaling in UUO rats was decreased following SSBE treatment. Thus, it was hypothesized that SSBE exerts renal anti-fibrotic effects via suppressing the hedgehog signaling pathway.

SSBE inhibits EMT induction and ECM accumulation in TGF-β1-treated RTECs. In UUO kidneys, upregulated

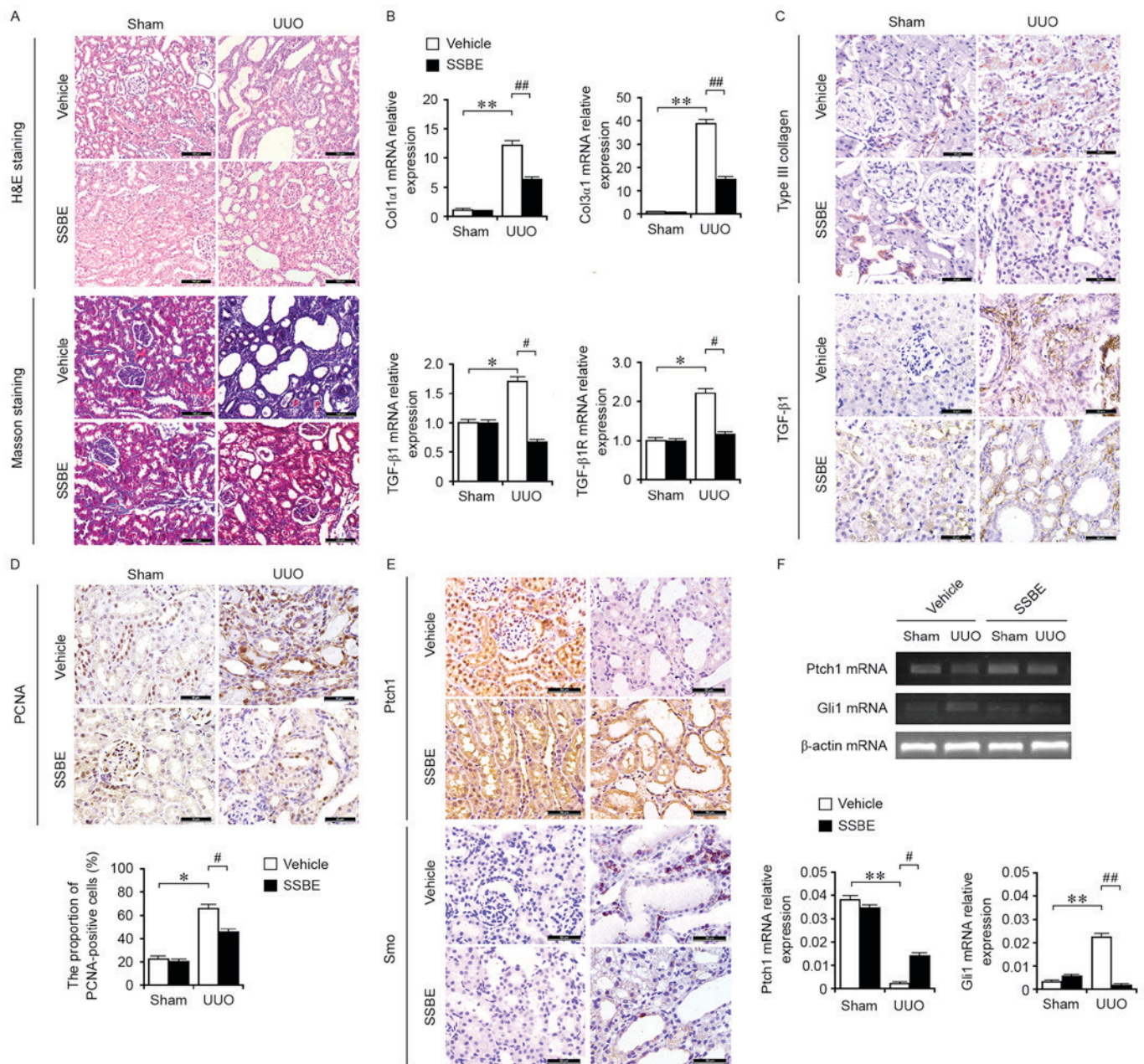


Figure 1. SSBE inhibits hedgehog signaling activity and alleviates interstitial fibrosis in UUO kidneys. (A) H&E and Masson trichrome staining indicated marked kidney injury and excessive accumulation of total collagen in UUO kidneys, but SSBE administration alleviated this effect. Scale bar, 100 μ m. (B) Enhanced mRNA expression levels of Col1 α 1, Col3 α 1, TGF- β 1 and TGF- β 1R in UUO kidneys, determined by reverse transcription quantitative polymerase chain reaction, were inhibited by SSBE treatment. (C) Immunohistochemical staining indicated upregulated expression of Col3 α 1 and TGF- β 1 in UUO kidneys, which were alleviated following SSBE administration. Scale bar, 50 μ m. (D) SSBE decreased PCNA expression in kidney tissues of UUO rats. Scale bar, 50 μ m. (E) SSBE administration inhibited UUO-induced downregulated protein expression levels of Ptch1, and upregulated expression of Smo. Scale bar, 50 μ m. (F) UUO decreased mRNA expression levels of Ptch1 and increased expression of Smo, but were inhibited by SSBE treatment. Data are presented as the mean \pm standard error. * P <0.05, ** P <0.01 vs. sham; # P <0.05, ## P <0.01 vs. vehicle. H&E, hematoxylin and eosin; UUO, unilateral ureteral obstruction; Col1 α 1, type I collagen; Col3 α 1, type III collagen; TGF- β 1, transforming growth factor- β 1; TGF- β 1R, transforming growth factor β 1 receptor; SSBE, *Sedum sarmentosum* Bunge; PCNA, proliferating cell nuclear antigen; Ptch1, protein patched homolog 1; Smo, smoothened.

expression levels of molecules involved in the activation of hedgehog signaling are primarily located around the tubules, which are rich in tubular epithelial cells (17). However, whether activation of hedgehog signaling is responsible for the proliferation of epithelial cells remains unknown. The present study investigated the effects of SSBE on EMT induction and ECM deposition in RTECs (NRK-52E cells) treated with TGF- β 1, an important inducer triggering EMT and ECM deposition. As expected, in TGF- β 1-treated NRK-52E

cells, upregulated expression of Col3 α 1 and the myofibroblast marker α -SMA, and downregulated expression of the epithelial marker E-cadherin, were observed (Fig. 2A). In addition, TGF- β 1 decreased the mRNA expression levels of ZO-1, and increased the expression of α -SMA, Col1 α 1 and Col3 α 1, compared with the control group (Fig. 2B). These TGF- β 1-mediated fibrotic alterations, including EMT induction and ECM accumulation, were inhibited by SSBE treatment in a dose-dependent manner; SSBE at the higher

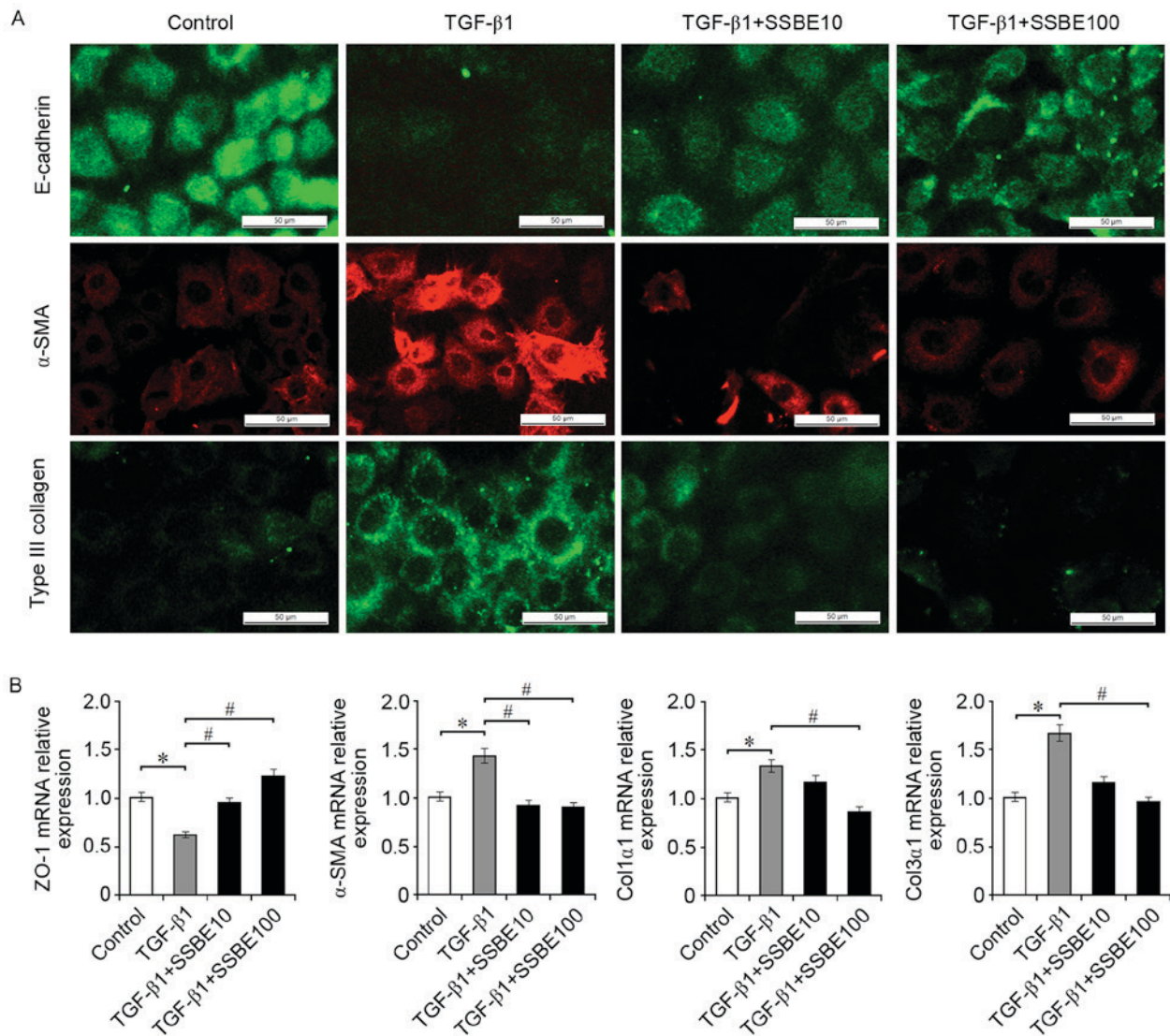


Figure 2. SSBE inhibits extracellular matrix accumulation in TGF- β 1-treated renal tubular epithelial cells. (A) Immunofluorescence staining indicated that SSBE treatment decreased TGF- β 1-mediated downregulated expression of E-cadherin, and upregulated expression of α -SMA and Col3 α 1 in NRK-52E cells. Scale bar, 50 μ m. (B) Reverse transcription quantitative polymerase chain reaction analysis demonstrated that mRNA expression levels of α -SMA, Col1 α 1, and Col3 α 1 were increased, and the expression of ZO-1 was decreased in TGF- β 1-treated cells; however, this effect was ameliorated following SSBE treatment. TGF- β 1, 5 ng/ml; SSBE10, 10 μ g/ml; SSBE100, 100 μ g/ml. Data are presented as the mean \pm standard error. *P<0.05 vs. control; #P<0.05 vs. TGF- β 1. SSBE, *Sedum sarmentosum* Bunge; Col1 α 1, type I collagen; Col3 α 1, type III collagen; TGF- β 1, transforming growth factor- β 1; α -SMA, α -smooth muscle actin; ZO-1, tight junction protein 1.

concentration (100 μ g/ml) had a stronger anti-fibrotic activity. However, SSBE at too high concentrations (>1,000 μ g/ml) inhibited cellular proliferation and induced apoptosis (data not shown), suggesting that an overdose of SSBE may have a cytotoxic effect.

SSBE inhibits TGF- β 1-induced activation of hedgehog signaling in RTECs. In addition to the induction of EMT and deposition of ECM, TGF- β 1 enhanced the activity of hedgehog signaling in NRK-52E cells. As presented in Fig. 3, upregulated expression of PCNA in association with activated hedgehog signaling was observed in TGF- β 1-treated NRK-52E cells. However, SSBE treatment effectively inhibited PCNA expression. Furthermore, SSBE downregulated the mRNA expression levels of Shh and Gli1 in NRK-52E cells after TGF- β 1 treatment (Fig. 3B and C), although the changes of Smo expression levels were not significant. Thus, this *in vitro*

experiment reconfirmed that inhibiting hedgehog activity may be an important molecular mechanism for the anti-fibrotic effect of SSBE on renal tissues *in vivo*.

SSBE inhibits AA-mediated over-activity of hedgehog signaling and ECM deposition. AA is regarded as a potent mutagen that induces significant cytotoxic effects on RTECs. *In vivo*, AA may cause a devastating renal disease called AA nephropathy, and the histopathology features interstitial matrix deposition and fibrosis (18). Therefore, the present study evaluated the effect of SSBE on RTECs following AA injury. The results demonstrated that SSBE treatment inhibited AA-induced overexpression of α -SMA and Col3 α 1 protein (Fig. 4A), but also decreased mRNA expression levels of TGF- β 1, α -SMA and Col1 α 1 (Fig. 4B). Furthermore, SSBE reduced the overactivation of hedgehog signaling in RTECs following AA injury by upregulating the mRNA expression

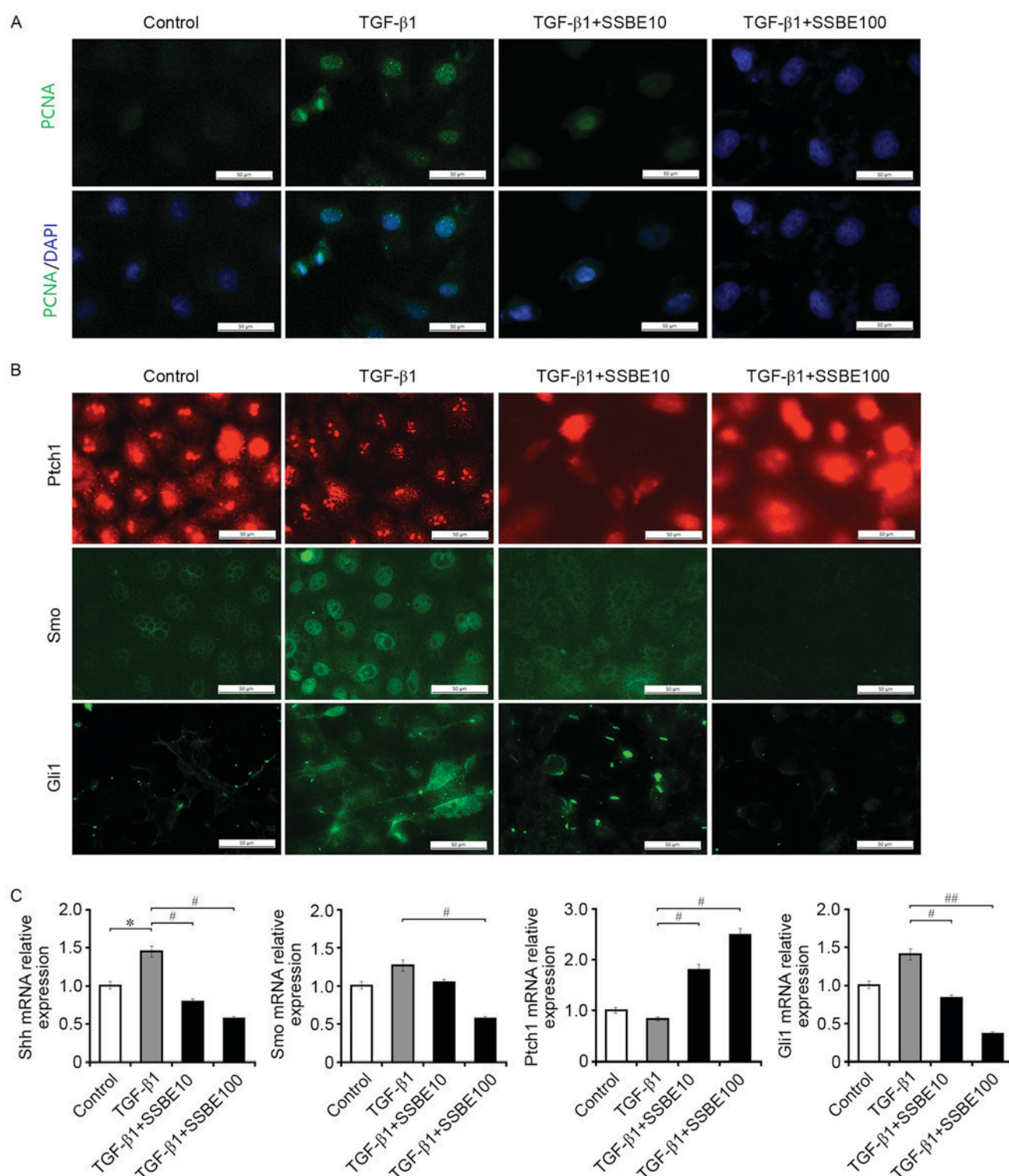


Figure 3. SSBE inhibits TGF- β 1-induced activation of hedgehog signaling in renal tubular epithelial cells. (A) SSBE inhibits TGF- β 1-induced PCNA expression in NRK-52E cells. Scale bar, 50 μ m. (B) SSBE inhibits TGF- β 1-induced upregulated expression of Smo and Gli1 in NRK-52E cells, and downregulated expression of Ptch1. Scale bar, 50 μ m. (C) The mRNA expression levels of Shh, Smo and Gli1 were increased, and the expression levels of Ptch1 were decreased, in TGF- β 1-treated cells. This effect was inhibited following SSBE treatment. Data are presented as the mean \pm standard error. TGF- β 1, 5 ng/ml; SSBE10, 10 μ g/ml; SSBE100, 100 μ g/ml. * P <0.05 vs. control; # P <0.05, ## P <0.01 vs. TGF- β 1. SSBE, *Sedum sarmentosum* Bunge; TGF- β 1, transforming growth factor- β 1; Smo, smoothened; Shh, sonic hedgehog; Ptch1, protein patched homolog 1; Gli1, Gli family zinc finger 1; PCNA, proliferating cell nuclear antigen.

of Ptch1 and downregulating mRNA and protein expression of Smo (Fig. 4A and C). Therefore, in an injured micro-environment, SSBE exhibits inhibitory activities on hedgehog signaling, resulting in the reduction of ECM deposition and a reduction in fibrosis.

Discussion

The present study examined the anti-renal fibrotic effects of SSBE *in vivo* and *in vitro*. In the kidney tissues of UUO rats, SSBE administration significantly alleviated tubular damage

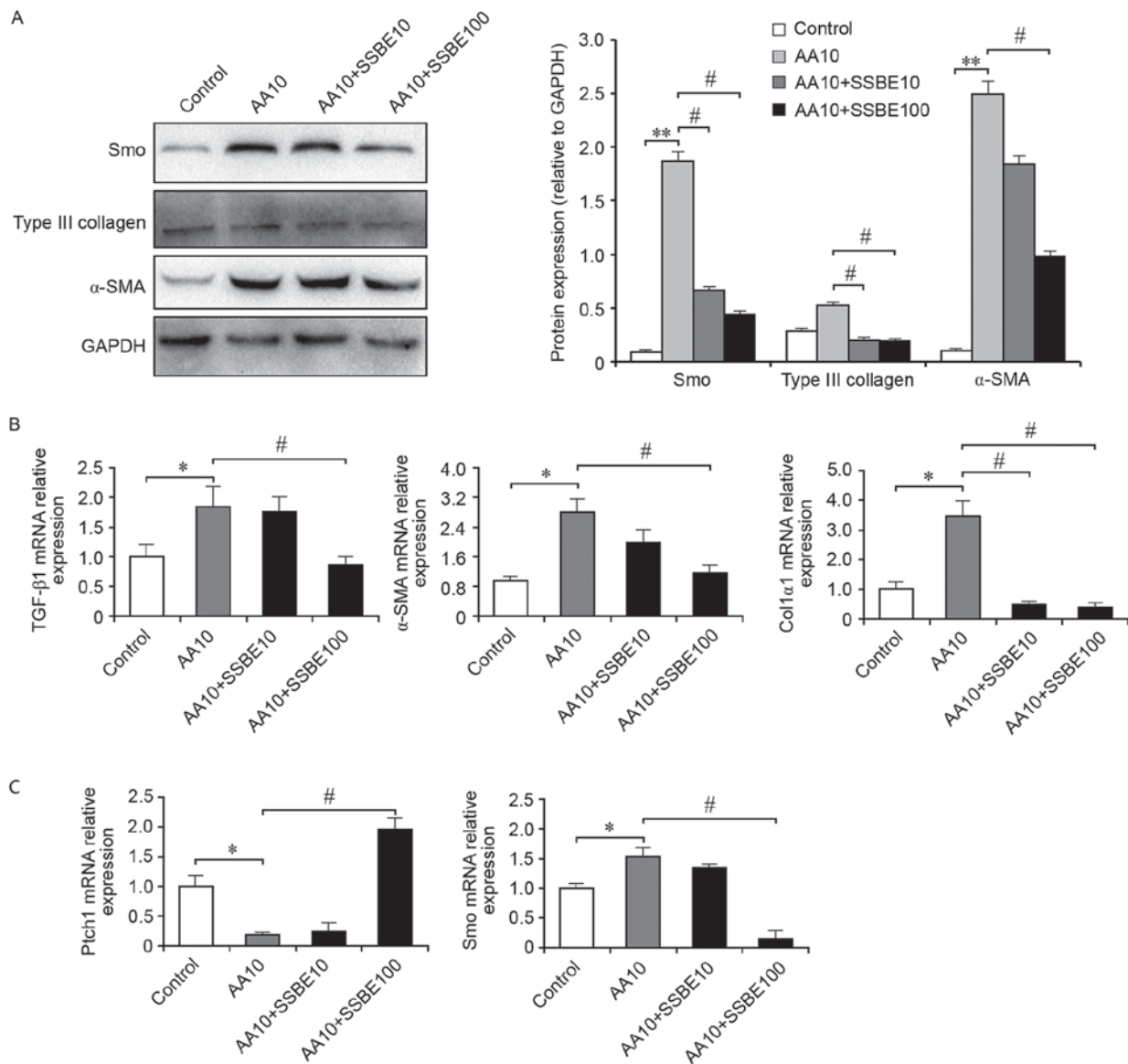


Figure 4. SSBE inhibits AA-mediated activation of hedgehog signaling and extracellular matrix deposition. (A) SSBE inhibits AA-induced overexpression of Smo, α -SMA and Col3a1 protein in NRK-52E cells, as assessed by western blot analysis. GAPDH served as an internal control. (B) Quantification of TGF- β 1, Col1a1 and α -SMA mRNA expression levels, as assessed by reverse transcription-quantitative polymerase chain reaction. (C) Quantification of Ptc1 and Smo mRNA expression levels. AA10, 10 μ g/ml; SSBE10, 10 μ g/ml; SSBE100, 100 μ g/ml. Data are presented as the mean \pm standard error. * P <0.05, ** P <0.01 vs. control; # P <0.05 vs. AA10. SSBE, *Sedum sarmentosum* Bunge; TGF- β 1, transforming growth factor- β 1; Smo, smoothened; Ptc1, protein patched homolog 1; Col1a1, type I collagen; α -SMA, α -smooth muscle actin; AA, aristolochic acid.

and interstitial fibrosis. In addition, SSBE effectively inhibited the formation of α -SMA-positive myofibroblasts and reduced excessive accumulation of ECM components in RTECs following exposure to the profibrotic factor TGF- β 1, or the noxious chemical AA.

These results indicated that the anti-fibrotic effect of SSBE on renal tissues may be associated with the inhibition of proliferation. SSBE treatment significantly decreased the expression levels of PCNA, a reference biomarker for cellular proliferation, not only in kidney tissues of UUO rats, but also in TGF- β 1-treated RTECs, suggesting that the inhibitory effect of SSBE on proliferation includes epithelial cells. The proliferation process of RTEC after injury involves regeneration and the EMT response (19,20). When the injury is moderate, limited and short-term cell death is controlled by the regenerative process, in which functional RTECs are

replaced by cells of the same lineage. When regeneration fails to keep pace with cell death, the injured RTECs may lose their polarity, develop the ability to migrate, and acquire improved plasticity. As a result, the trans-differentiation of RTECs is induced and the cells form α -SMA-positive myofibroblasts, leading to excessive accumulation of the fibrous matrix and the formation of a scar (21). The inhibition of proliferation in RTECs following SSBE treatment resulted in reduction of ECM accumulation and a decrease in fibrotic alterations. Thus, it is hypothesized that SSBE may be a potential agent for the treatment of fibrotic kidney disease. In addition, the inactivation of proliferation-associated signaling may be an important factor that mediates the inhibition of cellular proliferation.

Hedgehog signaling is a proliferation-associated pathway that serves a crucial role in the genesis and the development of several malignancies (22,23). Aberrant activation of hedgehog

signaling induces the expression of c-Myc and cyclin D1, resulting in the disorder in regulation of cell cycle (24,25). In tumor cells, hedgehog signaling is activated for various reasons, and induces cellular over-proliferation and malignant alterations. Similar to cancerogenesis, fibrogenesis may be an outcome of abnormal proliferation in certain tissue cells, such as RTECs, and activation of hedgehog signaling may contribute to this fibrotic fate. Numerous studies have confirmed that activated hedgehog signaling is involved in fibrogenesis of many tissues, including the liver and kidney (4,15). The present study also supported the conclusion that hedgehog signaling is activated during renal fibrogenesis, and then promotes the formation of myofibroblasts from RTECs via the EMT process. Treatment with SSBE downregulated the hedgehog signaling activity, resulting in the abolishment of EMT induction and ECM deposition. Thus, the renal anti-fibrotic effect of SSBE may occur through suppressing the activation of hedgehog signaling.

Previous pharmacological studies have revealed that SSBE possesses significant anti-inflammatory, antitumor and anti-viral infection activities (26,27). SSBE treatment exerts a marked inhibitory effect on lipopolysaccharide-induced nitric oxide production in RAW264.7 macrophage cells (26). In addition, in a hepatoma cell line, SSBE treatment inhibited proliferation and induced apoptosis through suppressing signal transducer and activator of transcription (STAT) phosphorylation (28). Furthermore, SSBE may relieve the symptoms of trinitrobenzene sulphonic acid-induced experimental colitis through reducing TGF- β 1 levels in T cells (29). STAT is a key transcription factor that regulates activation of the hedgehog signaling pathway (30,31). As a profibrotic factor *in vivo*, TGF- β 1 is regarded as an important inducer that triggers hedgehog signaling activation (19,21,32). Thus, these findings reconfirmed that hedgehog signaling may be involved in the anti-fibrotic effect of SSBE.

However, it should be noted that SSBE is a complex Chinese herb consisting of multiple active chemical constituents. The pharmacological function of SSBE may depend largely on the activities of these chemical constituents. Thus, further studies are required to clarify the potential molecular mechanism for each chemical constituent of SSBE, and to screen effective agents for fibrotic kidney disease.

In conclusion, these *in vitro* and *in vivo* experiments preliminarily demonstrated that SSBE treatment inhibited the hedgehog signaling pathway by reducing TGF- β 1 expression and abolishing the induction of EMT, resulting reduced accumulation of ECM components in the cortical interstitium. These results implicate SSBE as a potential therapeutic agent for the prevention of kidney fibrosis.

Acknowledgements

The present study was supported by the Natural Science Foundation of Zhejiang Province (grant no. LY17H050005) and the Natural Science Foundation of China (grant no. 81572087). The project was also supported by the Wenzhou Municipal Science and Technology Plan Project (grant no. Y20150037).

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