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Original Paper

Astragalus Inhibits Epithelial-to-Mesenchymal Transition of Peritoneal Mesothelial Cells by Down-Regulating β-Catenin

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Key Words
Peritoneal mesothelial cells • EMT • β-catenin • Astragalus

Abstract
Background/Aims: The epithelial-to-mesenchymal transition (EMT) of peritoneal mesothelial cells (PMCs) is a crucial event in the induction of peritoneal fibrosis (PF), in which canonical Wnt/β-catenin signaling participates. Smads signaling is reported to interact with β-catenin and synergistically regulates EMT. This study was aimed to reveal the effect of Astragalus on β-catenin in EMT of PMCs. ***Methods:*** To obtain the role of β-catenin in EMT, gene transfer into HMrSV5 cell line and rats has been achieved. After Astragalus treatment, EMT markers and signaling pathway-related indicators were detected by western blotting, immunofluorescence, immunohistochemistry, immunoprecipitation and real time-PCR. ***Results:*** β-catenin knockdown suppressed EMT of HMrSV5 cells. Astragalus alleviated EMT of PMCs characterized by increased E-cadherin and decreased α-SMA and Vimentin. In rat model of peritoneal dialysis (PD), Astragalus attenuated peritoneal thickening and fibrosis. Astragalus down-regulated β-catenin by stabilizing the Glycogen synthase kinase-3β (GSK-3β)/β-catenin complex and further inhibited the nuclear translocation of β-catenin. Meanwhile, Astragalus down-regulated β-catenin by enhancing Smad7 expression. Silencing Smad7 antagonized the EMT-inhibitory effect of Astragalus. ***Conclusion:*** Astragalus inhibits EMT of PMCs by down-regulating β-catenin. The modulation of β-catenin in peritoneum can be a novel tool to prevent PF.

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Introduction

Peritoneal dialysis (PD) is the renal replacement therapy for end-stage renal disease (ESRD). PD could be a feasible and safe alternative to hemodialysis and not only offers the advantages of being a less-invasive procedure and achieving a more continuous removal of waste products but also is beneficial for the retention of residual renal function. Current epidemiology indicates that more than 272, 000 patients receive PD worldwide, accounting for approximately 11% of the global dialysis population [1]. Unfortunately, the long-term instillation of PD fluid that is biologically incompatible, and recurrent peritonitis result in progressive peritoneal fibrosis (PF) and ultimately ultrafiltration failure.

The maintenance of normal peritoneal morphology and function is essential for patients on PD [2]. Epithelial-to-mesenchymal transition (EMT) has been widely considered a crucial process in PF. EMT is a reversible process by which epithelial cells are able to transform into cells with mesenchymal characteristics. Peritoneal mesothelial cells (PMCs) coexpress epithelial and mesenchymal markers under basal conditions, due to their mesodermal origin, which explains their enhanced plasticity [3]. PMCs that have undergone EMT exhibit an augmented ability to detach from basement membranes and invade into the submesothelial zone, leading to extracellular matrix (ECM) deposition and collagen formation. EMT is characterized by the loss of epithelial differentiation markers and the induction of mesenchymal markers. Previous studies reported that multiple signaling, including TGF-β1/Smad, Wnt/β-catenin, Notch, Hedgehog and other pathways, participate in regulating the process of EMT [4]. We focus on Wnt/β-catenin signaling and the molecular mechanism by which that pathway triggers EMT to illustrate a potential method for inhibiting PF.

β-catenin is the key component of the Wnt/β-catenin signaling, which has been demonstrated to regulate cell proliferation, differentiation and apoptosis [5]. β-catenin is a dual-function protein that is involved in EMT. This protein improves cell-cell adhesion by connecting cadherins to the cytoskeleton. In addition, β-catenin serves as a transcription factor to activate downstream target genes [6]. Some evidences indicate that altered β-catenin expression, activation and subcellular localization are implicated in EMT and fibrosis [7, 8]. Indeed, Wnt/β-catenin can be regulated by other signaling, such as the TGF-β pathway [9, 10]. The Wnt/β-catenin and TGF-β pathways synergize in mesenchymal-derived cells of various organs to promote ECM deposition and fibroblast activation [11]. Both the Wnt/β-catenin and TGF-β pathways are activated in EMT, but how these crucial signaling respond in injured PMCs to impact PF is unknown.

Astragalus (*Astragalus membranaceus*) has been used in Traditional Chinese Medicine for the treatment of various diseases for over two thousand years. This organism plays effective roles in reducing inflammation, attenuating insulin resistance in obesity and diabetes, and modulating the immune response, with minor side effects [12, 13, 14]. Recent studies have reported the anti-fibrogenic effect of Astragalus on cardiac fibroblasts, liver fibrosis, and renal interstitial fibrosis [15, 16, 17]. In our previous studies, we revealed that adding Astragalus to the dialysate enhanced the water clearance of the rat peritoneum and inhibited PD fluid-induced PF via TGF-β1 signaling [18]. Astragaloside IV, a saponin purified from Astragalus, could prevent TGF-β1-induced EMT in PMCs by up-regulating Smad7 [19]. Based on the potential relevance between the Wnt/β-catenin and TGF-β pathways, we focus on the effect of Astragalus on β-catenin. Intriguingly, we found that Astragalus could inhibit β-catenin expression, but the molecular mechanism of β-catenin down-regulation remained to be elucidated. Here, we verified that Astragalus ameliorates PF and determined that Astragalus exhibits the most potent effects against EMT in PMCs, through a molecular mechanism involving the down-regulation of β-catenin.

Materials and Methods

Materials

DMEM basic was supplied by HyClone (Logan, UT, USA). Fetal calf serum (FCS) and penicillin/streptomycin were supplied by Invitrogen (Carlsbad, CA, USA). Alexa Fluor 488 Donkey anti-Rabbit IgG, RIPA lysis buffer, phosphatase inhibitor, protease inhibitor, NE-PER nuclear and cytoplasmic extraction reagents, and BCA Protein Assay Kit were supplied by Thermo Fisher Scientific (Waltham, MA, USA). The TRIzol reagent was purchased from Life Technologies (Gaithersburg, MD, USA). The PrimeScript RT Master Mix and TB Green Premix Ex Taq II were purchased from Takara Bio Group (Dalian, China). The HiPerFect transfection reagent was purchased from Qiagen (Germany). 4',6-Diamidino-2-phenylindole (DAPI) was purchased from Beyotime Biotechnology (Shanghai, China). Protein-A/G-Sepharose was purchased from GE Healthcare (Piscataway, NJ, USA). TGF-β1 was supplied by PeproTech (Rocky Hill, NJ, USA). Standard PD fluid (Dianeal PD-2 peritoneal dialysis solution with 4.25% dextrose, pH 5.2) was supplied by Baxter HealthCare (Deerfield, IL, USA). Astragalus was obtained from Chiatai Qingchunbao Pharmaceutical Co., Ltd. (Hangzhou, China), containing 2 g/mL of crude drug, and the main constituents are polysaccharides, saponins (astragalosides I to IV), flavonoids (calycosin, formononetin 7-O-glucoside, calycosin 7-O-glucoside) and amino acids. Adenovirus-associated virus (AAV)-Smad7 and AAV-Smad7-siRNA were obtained from Genechem Co., Ltd. (Shanghai, China). The anti-E-cadherin, anti-β-catenin, anti-phospho-β-catenin (Ser552), anti-Smad2/3, anti-Smad4, anti-c-myc, anti-CyclinD1, anti-GSK-3β, anti-phospho-GSK-3β (Ser9), anti-β-actin, anti-GAPDH, anti-Histone H3 antibodies, SignalSilence β-catenin siRNAs (#6225, #6238), and secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit and anti-mouse IgG were obtained from Cell Signaling Technology (Boston, MA, USA). The anti-Collagen I, anti-Vimentin and anti-α-SMA antibodies were obtained from Abcam (Cambridge, UK). The anti-Smad7 antibody and Smad7 siRNA (sc-36508) were obtained from Santa Cruz Biotechnology (Texas, USA). The ECL system was obtained from Millipore (Bedford, USA).

Cell culture and treatment with reagents

Human peritoneal mesothelial cells (HPMCs, HMrSV5) (ATCC) were supplied by Jennio Biotech Co., Ltd. (Guangzhou, China) and cultured in DMEM basic supplemented with 10% FCS (v/v) and 1% penicillin-streptomycin (v/v) in a humidified atmosphere with 5% CO_2 at 37°C. The culture medium was replaced every 2 days. All experiments were carried out 24-48 h after the cells were cultured in culture plates. The cells were permitted to attach for 24 h and grow to 75% confluence. To induce EMT, HMrSV5 cells were treated with TGF-β1 at different doses and action times. Astragalus (2 g/mL) was added to the cell culture system, with the final concentration ranging from 20 to 800 mg/mL due to different experimental designs.

Cell viability assay

Cell viability was measured in a 96-well plate using a quantitative colorimetric assay. The cells were cultured in 96-well plates in triplicate with various treatments. A CCK-8 solution (10 µL) was applied to each well, and all wells were incubated for another 1 h at 37°C. Absorbance was measured at 450 nm (Bio-Rad 550, USA).

Cell transient transfection

HMrSV5 cells were transfected with 100 nM β-catenin or Smad7 siRNA using the HiPerFect transfection reagent according to the manufacturer's instructions. Forty-eight hours after siRNA treatment, the cells were stimulated as indicated for an additional 24 h with treatment reagents and were collected.

PF rat model, Smad7 gene transfection in rats and treatment with reagents

Male Sprague-Dawley rats, 180-200 g in weight, were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The PD fluid rat model was developed by daily intraperitoneal injection with standard PD fluid at a dose of 100 mL/kg/day for 35 days. Rats in the Astragalus groups were subjected to intraperitoneal injection with 4, 000 mg/kg/day of Astragalus with or without PD fluid. The Smad7 knockdown and overexpression rat model was established by injecting AAV-Smad7 siRNA or AAV-Smad7 once into the tail vein at a dose of 1.00E+11 v.g/mL for 21 days before intraperitoneal injection of PD fluid and Astragalus. Under the guidance of the Animal Care and Use Committee, all of the animals were treated

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humanely and were randomly divided into six groups, as follows: (1) Control (n = 6); (2) Peritoneal dialysis (injected intraperitoneally with PD fluid, n = 6); (3) Astragalus alone (injected intraperitoneally with an equal volume of saline + Astragalus, n = 6); (4) Astragalus treatment (injected intraperitoneally with PD fluid + Astragalus, n = 6); (5) Smad7 knockdown (AAV-Smad7 siRNA + PD fluid + Astragalus, n = 6); and (6) Smad7 overexpression (AAV-Smad7 + PD fluid, n = 6). After treatment, the abdomens of the rats were opened with a midline incision. Next, parietal peritoneum and subpyloric omentum samples that were 3 mm × 1 mm × 1 mm in size were removed for further examination.

Western blotting

The total protein from rat peritoneum or HMrSV5 cells was extracted using ice-cold RIPA buffer containing PMSF, phosphatase inhibitor and protease inhibitor cocktail. Nuclear and cytoplasmic proteins were obtained by NE-PER nuclear and cytoplasmic extraction reagents. The BCA protein assay was used to detect the protein concentration. Electrophoresis and western blotting analysis were performed as described previously [19].

Real-time PCR

The RNA from rat peritoneum or HMrSV5 cells was extracted using TRIzol reagent according to the manufacturer's instructions. Reverse transcription of RNA was achieved using the PrimeScript RT Master Mix and TB Green Premix Ex Taq II. Real-time PCR was performed using the ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The PCR primers were designed as follows:

E-cadherin:5'-CCACCAGATGACGATACCCG-3'5'-GAATCACTTCCGGTCTGGCA-3';Vimentin:5'-CAGTCACTCACCTGCGAAGT-3'5'-AGTTAGCAGCTTCAAGGGCA-3';α-SMA:5'-CATCACCAACTGGGACGACA-3'5'-TCCGTTAGCAAGGTCGGATG-3';β-catenin:5'-CCCTGACAGAGTTGCTCCAC-3'5'-CCGAAAGCCGTTTCTTGTAG-3';Smad7:5'-CGTGCAGATTAGCTTCGTGA-3'5'-GAGACTACCGGCTGTTGAAGA-3';Snail:5'-CGAGTGGTTCTTCTGCGCTA-3'5'-GGGCTGCTGGAAGGTAAACT-3';Slug:5'-AGCACATTGCATCTTTTCTTACT-3'5'-GAAAGACATGGGACACGCAC-3';GAPDH:5'-CCTTCATTGACCTCAACTACATG-3'5'-CTTCTCCATGGTGGTGAAGAC-3'.

All mRNA quantification data were normalized to GAPDH as an endogenous control for mRNA detection. The data were processed using the $2^{-\Delta\Delta Ct}$ method.

Immunofluorescence assay

The cells (5×10^4/well) were cultured in an 8-well glass Nunc Lab-Tek chamber slide and were treated with TGF-β1 with or without Astragalus. After 24 h treatment, the slides were washed, and the cells were fixed, permeabilized and blocked. Next, the cells were incubated overnight with the primary antibody at 4°C, followed by a fluorescent secondary antibody for 1 h at room temperature and staining with DAPI for 5 min. Images were acquired with a Zeiss AX10 fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Coimmunoprecipitation (Co-IP)

The cells were lysed in RIPA buffer and harvested by centrifugation at 1500 g for 5 min. The cellular lysates were homogenized, cleared, and immunoprecipitated by an anti-β-catenin antibody at 4°C overnight. Immune complexes were precipitated with protein-A/G-Sepharose and incubated for 2 h at 4°C with gentle shaking on a rotator. Then, the beads were washed three times with ice-cold RIPA buffer. Another washing step with the indicated buffer followed. The samples were run on columns to allow the separation of the beads and precipitated proteins. The proteins were eluted from the beads by boiling in SDS-PAGE sample buffer for 3 min and subjected to western blotting analysis.

Histology and immunohistochemistry (IHC)

Parietal peritoneum samples were fixed in 10% neutral-buffered formalin for 24 h, dehydrated successively in a graded alcohol series (75%, 85%, 95% and 100%, v/v) and embedded in paraffin. The samples were sectioned at a 5 μm thickness. For histological examination, the sections were stained with hematoxylin-eosin (HE) and Masson's trichrome to quantify the pathological condition and peritoneum thickness. For IHC, the sections were stained with anti-Collagen I, anti-E-cadherin, anti-Vimentin, anti-α-SMA, anti-β-catenin and anti-Smad7 antibodies and then incubated with the appropriate secondary

antibody. Protein expression was detected with a DAB kit. Images were obtained using an Olympus BX45 inverted microscope.

Statistical analysis

All data from at least three independent experiments were expressed as the means ± standard error of the mean (SEM) and were analyzed using one-way ANOVA with SPSS 19.0 statistical software. Values of $P<0.05$ were considered statistically significant.

Results

Astragalus regulates the expression of EMT markers in HPMCs

TGF-β1 is the primary pathological factor causing fibrosis. In HMrSV5 cells, Astragalus reversed TGF-β1-induced EMT. First, the non-toxic nature of Astragalus was determined using a CCK-8 assay. Treatment of the cells with up to 800 mg/mL of Astragalus for 72 h had almost no effect on cell viability (Fig. 1A). Subsequently, we observed the effect of TGF-β1 based on the dose and action time. TGF-β1 treatment at 10 ng/mL for 24 h induced a marked reduction of the epithelial marker E-cadherin and a induction of the myofibroblast markers Vimentin and α-SMA, whereas Astragalus (800 mg/mL) effectively improved these abnormal expression levels, as indicated by western blotting (Fig. 1B-D). Morphologically, TGF-β1-treated HMrSV5 cells developed elongation, branching and the loss of the cobblestone-like appearance, whereas Astragalus treatment reduced these mesenchymal- or fibroblast-like changes (Fig. 2A). Immunofluorescence assays further indicated a significant increase in the expression of E-cadherin and a decrease in the expression of Vimentin and α-SMA after treatment with Astragalus (Fig. 2B).

Astragalus down-regulates β-catenin and inhibits β-catenin nuclear translocation in HPMCs

Some studies reported that the up-regulation of β-catenin promotes EMT [20]. Therefore, we assessed the effect of Astragalus on β-catenin. Activated Wnt/β-catenin signaling is characterized by increased expression and nuclear translocation of β-catenin [21]. As shown in Fig. 3A-B, after treatment with TGF-β1 at different doses and action times, the expression of β-catenin, c-myc and Cyclin D1, the target proteins of the canonical Wnt pathway, was strongly increased after treatment with a dose of 10 ng/mL for 24 h. Astragalus distinctly down-regulated the TGF-β1-induced induction of β-catenin, as well as c-myc and Cyclin D1 (Fig. 3C). Indeed, β-catenin acts a transcription factor together with T-cell factor/lymphoid enhancer factor (TCF/LEF) to initiate downstream gene transcription. Based on this critical mechanism of signal transduction, we next explored the translocation of β-catenin during EMT and the effect of Astragalus. Since the duration of TGF-β1 treatment was extended, the β-catenin level in the nucleus was increased and peaked at 24 h but subsequently declined, a finding that was consistent with our EMT model (Fig. 4A). Astragalus treatment reduced nuclear β-catenin in a dose-dependent manner, which correlated with a dose-dependent β-catenin reduction in the cytoplasm (Fig. 4B). An immunofluorescence assay also demonstrated that enhanced staining of nuclear β-catenin was induced by TGF-β1 stimulation and significantly reduced by Astragalus treatment (Fig. 4C).

β-catenin is involved in the EMT-inhibitory effect of Astragalus in HPMCs

To confirm that Astragalus inhibits EMT in HPMCs via the down-regulation of β-catenin, we established β-catenin knockdown HMrSV5 cells. As shown in Fig. 5A, HMrSV5 cells received either a negative control siRNA or a siRNA specific for β-catenin before TGF-β1 treatment. The results showed that β-catenin knockdown attenuates TGF-β1-induced EMT. β-catenin knockdown can also ameliorate morphological alterations caused by TGF-β1 treatment (Fig. 5B). In addition, TGF-β1 treatment up-regulated the EMT transcription factors Snail and Slug at mRNA level, but this effect was partially reversed by the knockdown

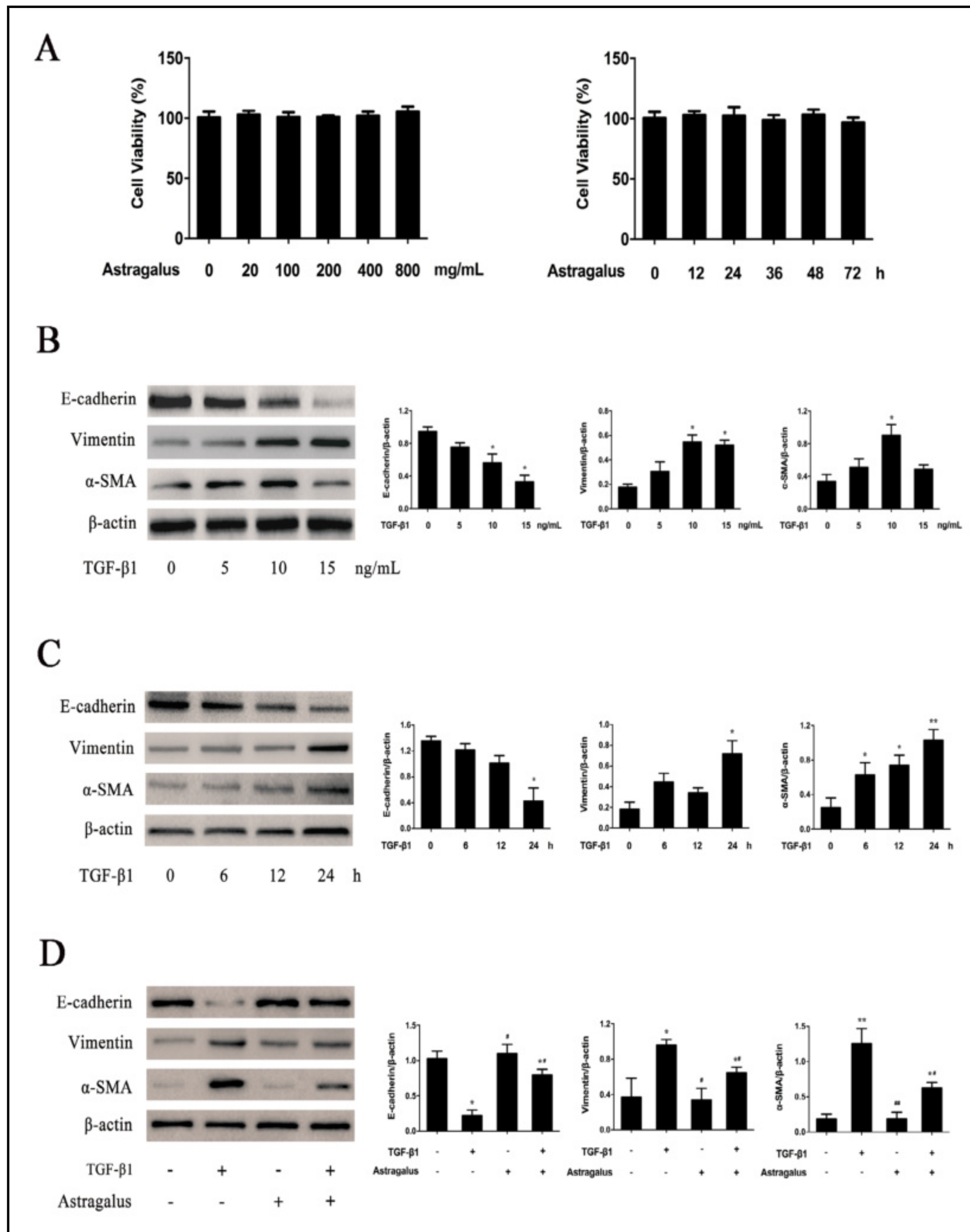

Fig. 1. Effect of Astragalus on TGF-β1-induced HPMCs EMT. (A) HMrSV5 cells were treated with different doses of Astragalus (0, 20, 100, 200, 400 and 800 mg/mL) for 24 h or with 800 mg/mL of Astragalus for different times (0, 12, 24, 36, 48 and 72 h). Neither strengthened cell proliferation nor apoptosis was observed. (B) HMrSV5 cells were treated with TGF-β1 at various concentrations (0, 5, 10, 15 ng/mL) for 24 h and subjected to western blotting for EMT markers. (C) HMrSV5 cells were treated with TGF-β1 (10 ng/mL) for various times (0, 6, 12 and 24 h) and subjected to western blotting for EMT markers. (D) HMrSV5 cells were exposed to Astragalus (800 mg/mL) with or without TGF-β1 (10 ng/mL) and subjected to western blotting for EMT markers. β-actin was used as a loading control. Data are expressed as mean±SEM, $^{*}p<0.05$ vs. control; $^{**}p<0.01$ vs. control; $^{\#}p<0.05$ vs. TGF-β1 treatment; $^{\#\#}p<0.01$ vs. TGF-β1 treatment.

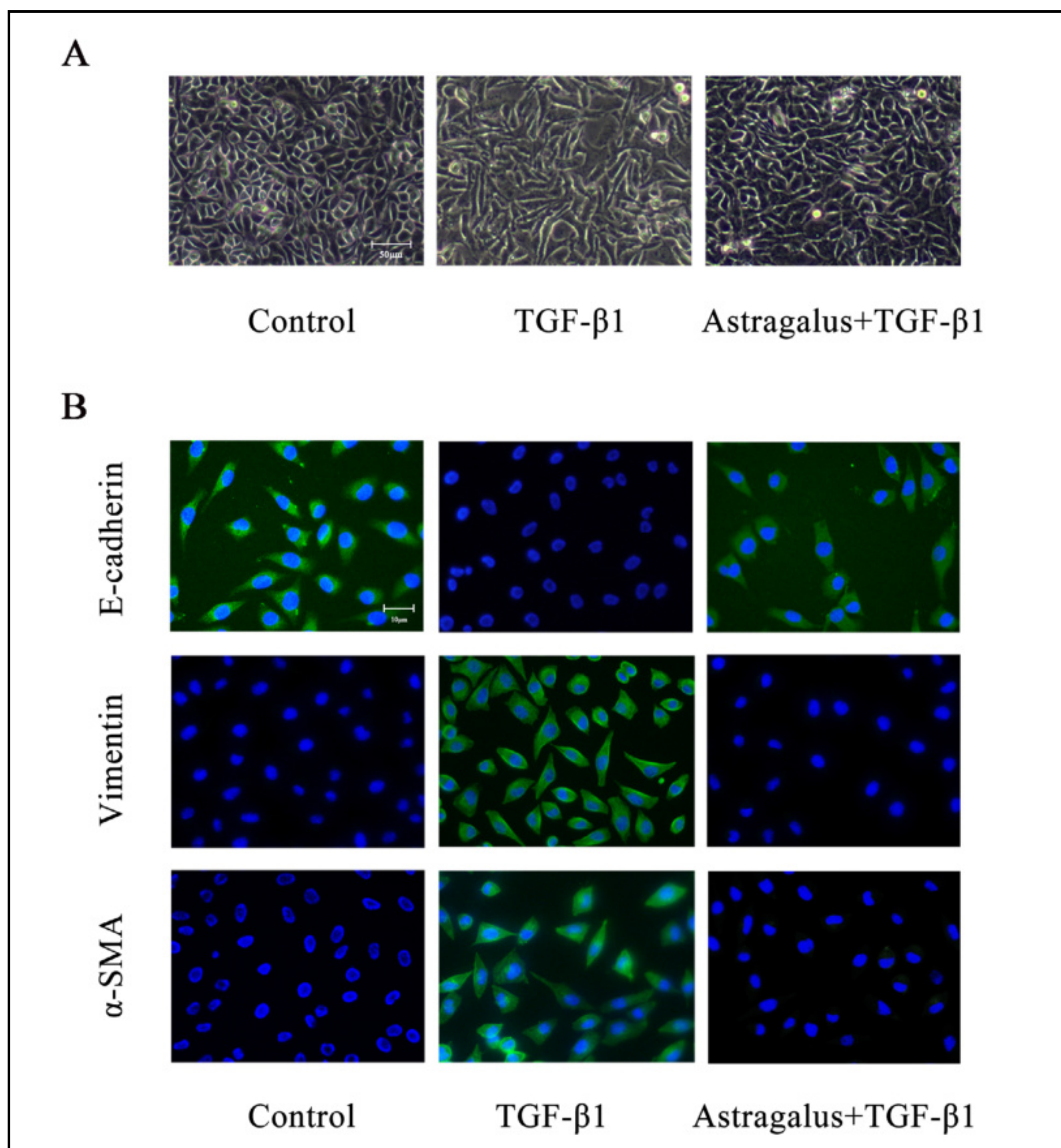

Fig. 2. Astragalus affects TGF-β1-induced HPMCs EMT. (A) HMrSV5 cells were treated with TGF-β1 (10 ng/mL) or Astragalus (800 mg/mL), the morphologic alterations of HMrSV5 were observed using a microscope (scale bar=50 μm). (B) HMrSV5 cells were exposed to Astragalus (800 mg/mL) and TGF-β1 (10 ng/mL) and subjected to fluorescence microscopy to visualize EMT markers (scale bar=10 μm).

of β-catenin (Fig. 5C). Importantly, Astragalus could further inhibit EMT after β-catenin siRNA treatment (Fig. 5D). These results suggested that the inhibition of EMT by Astragalus in HMrSV5 cells is dependent on β-catenin.

Astragalus down-regulates β-catenin by promoting the GSK-3β/β-catenin complex in HPMCs

In the canonical Wnt pathway, when intracellular signaling events are triggered by cytokines, the formation of a complex between GSK-3β and β-catenin is inhibited, ultimately resulting in the dephosphorylation of β-catenin. This dephosphorylation leads to the stabilization and nuclear transcription of β-catenin. Thus, the Co-IP assay was performed to determine whether Astragalus alters the binding of the GSK-3β and β-catenin proteins. The cells were treated with 10 ng/mL of TGF-β1 with or without 800 mg/mL of Astragalus. Fig.

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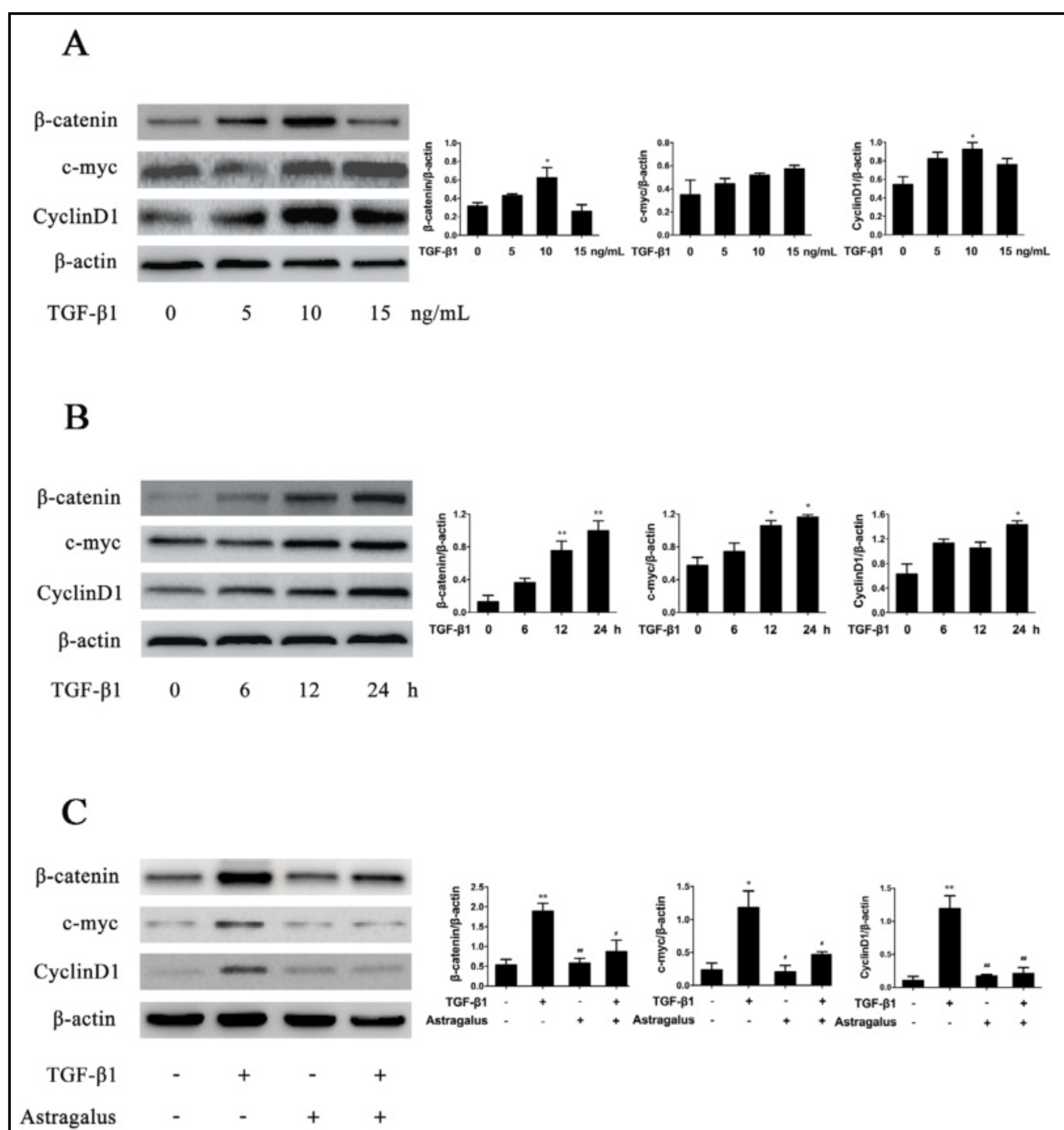

Fig. 3. Effect of Astragalus on β-catenin in HPMCs. (A) HMrSV5 cells were treated with TGF-β1 at various concentrations for 24 h and subjected to western blotting for β-catenin and the target proteins of the canonical Wnt/β-catenin pathway (c-myc and Cyclin D1). (B) HMrSV5 cells were treated with TGF-β1 (10 ng/mL) for various times and subjected to western blotting for β-catenin, c-myc and Cyclin D1. (C) HMrSV5 cells were exposed to Astragalus (800 mg/mL) with or without TGF-β1 (10 ng/mL) and subjected to western blotting for β-catenin, c-myc and Cyclin D1. Data are expressed as the mean±SEM, $^{*}p<0.05$ vs. control, $^{**}p<0.01$ vs. control; $^{\#}p<0.05$ vs. TGF-β1 treatment; $^{\#\#}p<0.01$ vs. TGF-β1 treatment.

6A showed that TGF-β1 inhibited GSK-3β binding to β-catenin, whereas Astragalus enhanced this cohesion and further down-regulated β-catenin. Indeed, normally in the cytoplasm, β-catenin is constitutively phosphorylated by GSK-3β. However, the phosphorylated GSK-3β in the cytoplasm, which has been inactivated, cannot phosphorylate β-catenin, increasing its cytoplasmic stimulation. Therefore, western blotting was performed to determine the effect of Astragalus on the expression of GSK-3β and β-catenin in the cytoplasm, as well as the expression of β-catenin in the nucleus. As shown in Fig. 6B-C, TGF-β1 significantly enhanced the phosphorylation of GSK-3β without affecting the total cytoplasmic GSK-3β and also decreased phosphorylated β-catenin. After Astragalus treatment, the phosphorylation of GSK-3β in the cytoplasm was inhibited, which correlated negatively with an increased

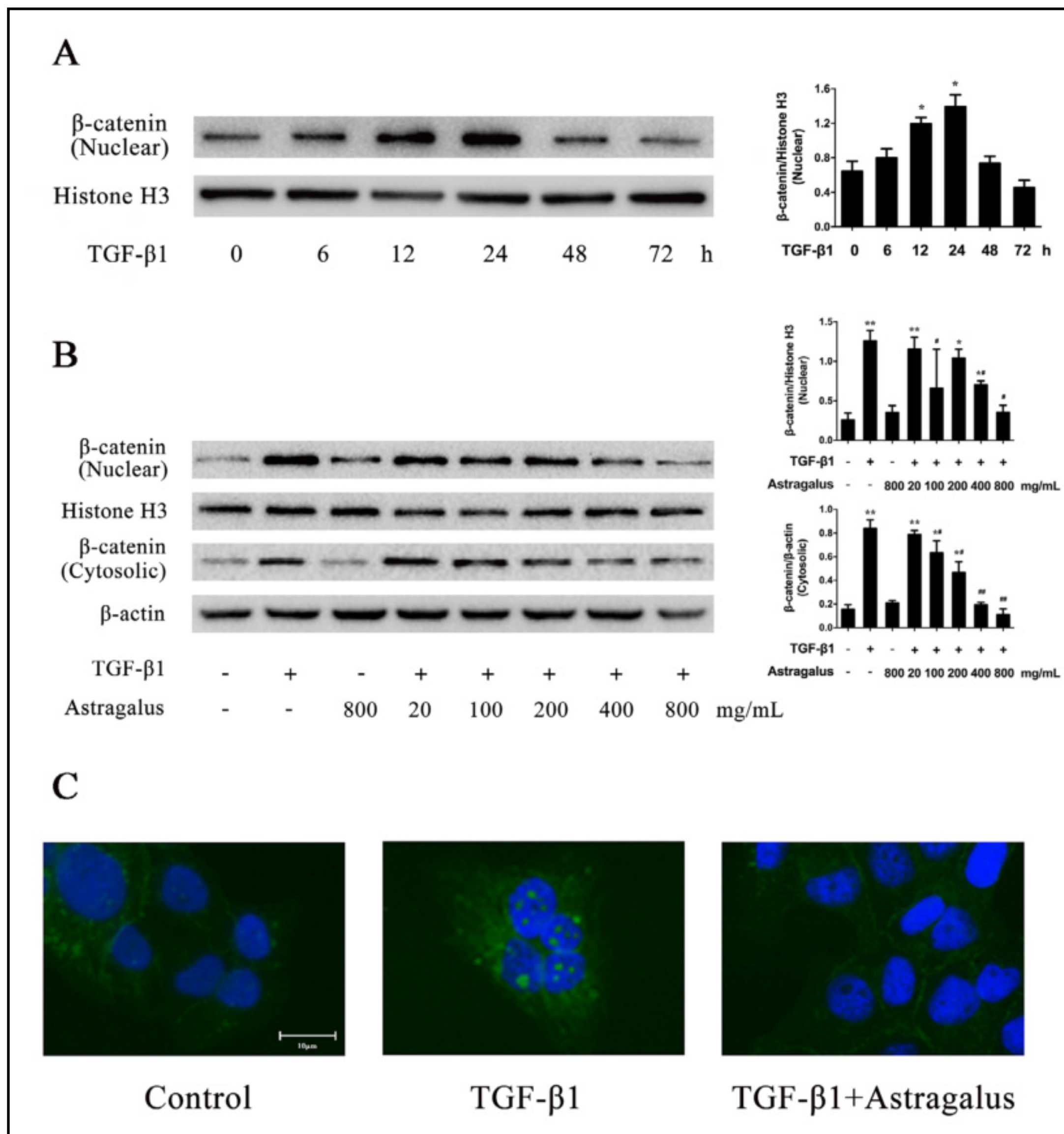

Fig. 4. Effect of Astragalus on β-catenin nuclear translocation in HPMCs. (A) HMrSV5 cells were treated with TGF-β1 (10 ng/mL) for various times (0, 6, 12, 24, 48 and 72 h), the expression of β-catenin in the nucleus was detected by western blotting. Histone H3 was used as the loading control for the nucleus. (B) After treatment with various doses of Astragalus (20, 100, 200, 400 and 800 mg/mL) with or without TGF-β1 (10 ng/mL), nuclear and cytosolic β-catenin expression was detected by western blotting. Histone H3 and β-actin were used as the loading controls for the nucleus and cytosol, respectively. Data are expressed as the mean±SEM, *$p<0.05$ vs. control, **$p<0.01$ vs. control; #$p<0.05$ vs. TGF-β1 treatment; ##$p<0.01$ vs. TGF-β1 treatment. (C) Immunofluorescence assays were performed to observe the subcellular translocation of β-catenin. β-catenin expression in the membrane, cytoplasm and nucleus were visualized based on the green fluorescent signal. The image was obtained by fluorescence microscopy (scale bar=10 μm).

level of phosphorylated β-catenin. Additionally, Astragalus reversed the increasing level of β-catenin in the nucleus induced by TGF-β1 (Fig. 6D). These results suggested that Astragalus down-regulates β-catenin by promoting the GSK-3β/β-catenin complex, which both increases the degradation of β-catenin in the cytoplasm and suppresses the nuclear transcription of β-catenin.

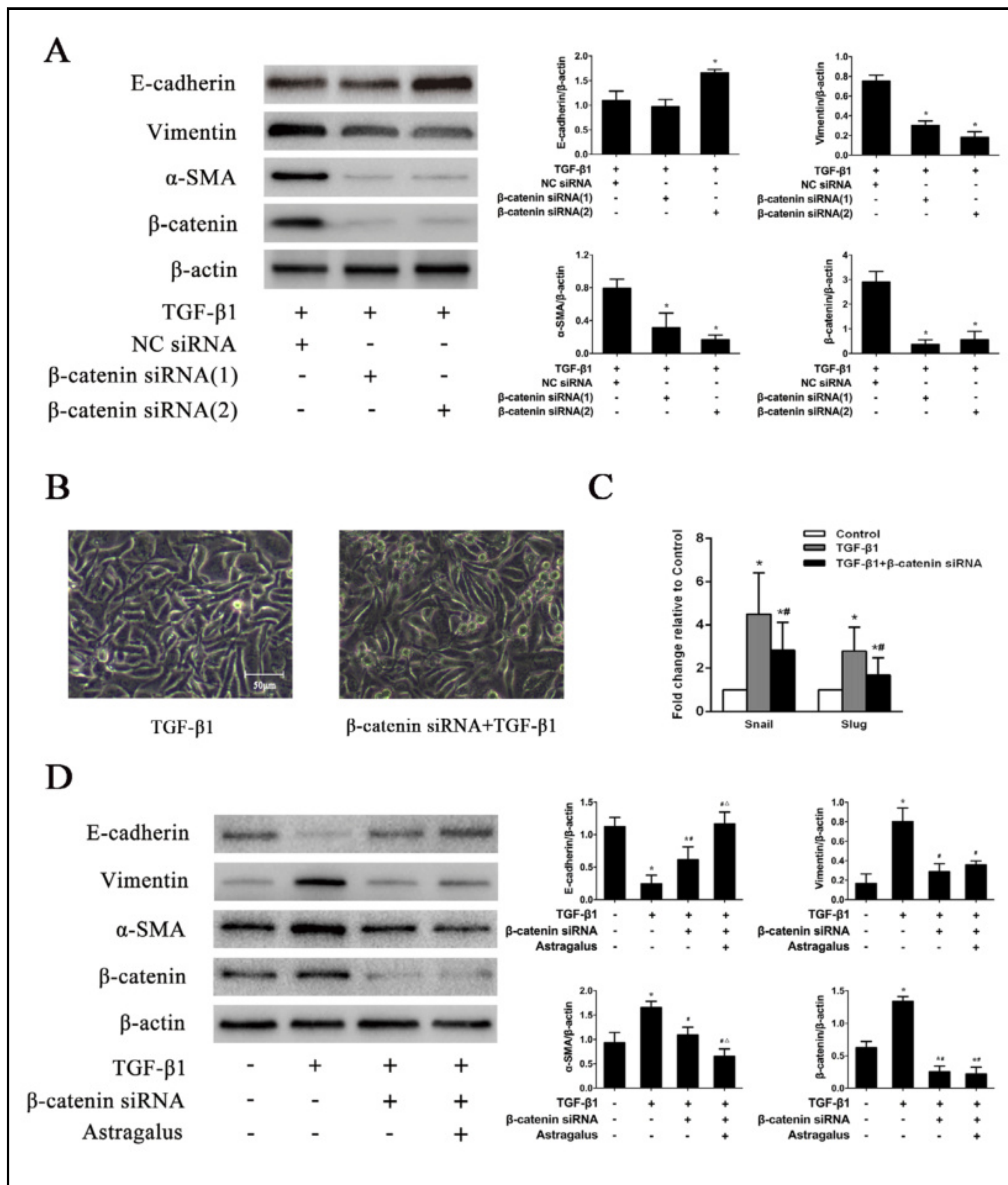

Fig. 5. β-catenin is involved in the EMT-inhibitory effect of Astragalus in HPMCs. (A) HMrSV5 cells were transiently transfected with the SignalSilence β-catenin Control siRNA (NC siRNA), siRNA(1) and siRNA(2) for 48 h and subsequently treated with TGF-β1 (10 ng/mL) for 24 h. β-catenin, E-cadherin, Vimentin and α-SMA were analyzed by western blotting. Data are shown as the mean±SEM, $^{*}p<0.05$ vs. TGF-β1 and NC siRNA treatment. (B) TGF-β1 induced a morphologic change in HMrSV5 from a cuboidal, cobble-stone appearance to a fibroblastic morphology, while β-catenin knockdown attenuated these changes (scale bar = 50μm). (C) HMrSV5 cells were exposed to TGF-β1 (10 ng/mL) and β-catenin siRNA (100 nM) and subjected to real-time PCR to measure mRNA levels of the EMT transcription factors Snail and Slug. Data are shown as the mean±SEM, $^{*}p<0.05$ vs. control, $^{\#}p<0.05$ vs. TGF-β1 treatment. (D) HMrSV5 cells were treated with β-catenin siRNA for 48 h and incubated with Astragalus (800 mg/mL) and TGF-β1 (10 ng/mL). Western blotting was performed to detect EMT markers and β-catenin expression. Data are shown as the mean±SEM, $^{*}p<0.05$ vs. control; $^{\#}p<0.05$ vs. TGF-β1 treatment; $^{\Delta}p<0.05$ vs. TGF-β1 and β-catenin siRNA treatment.

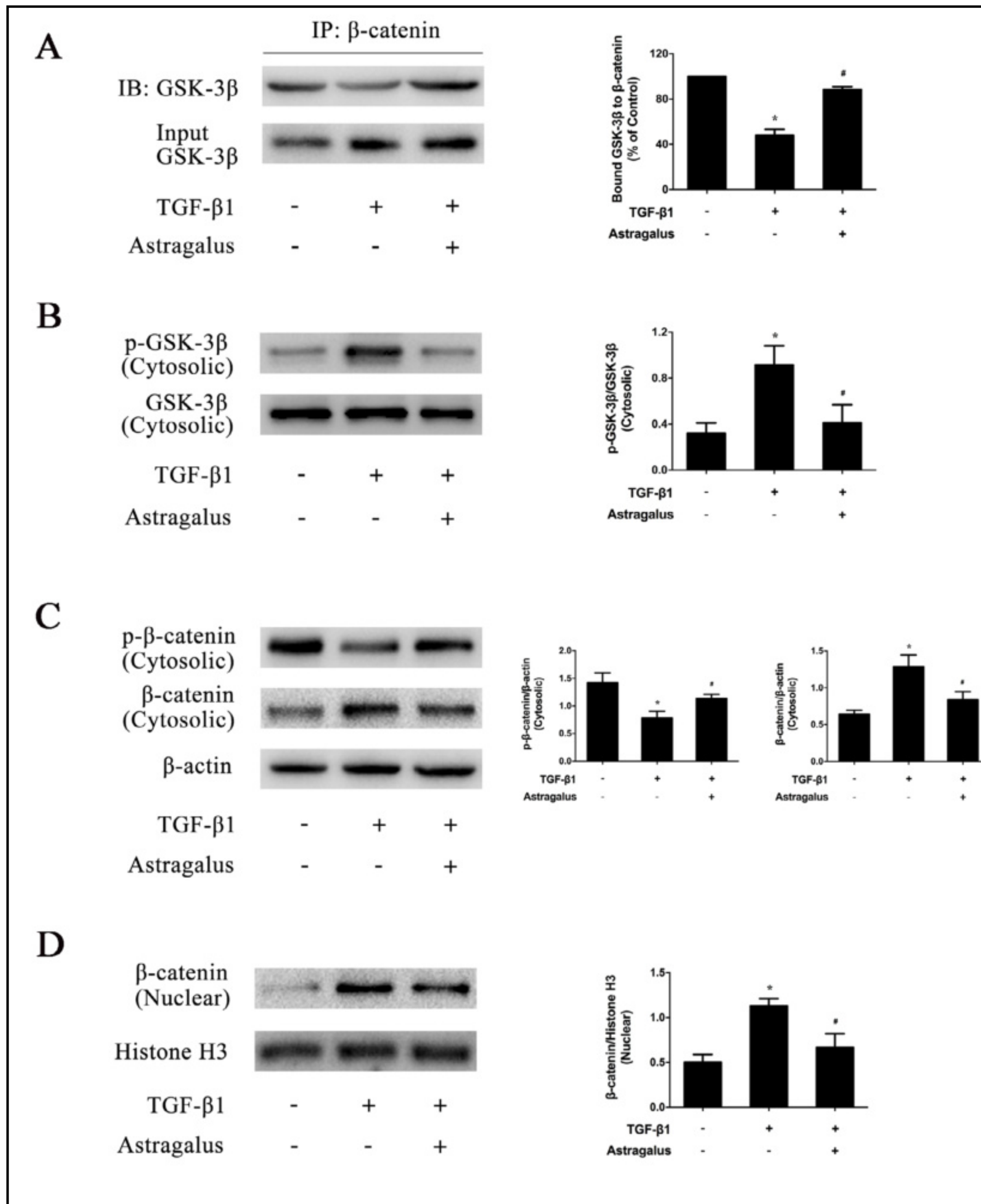

Fig. 6. Astragalus down-regulates β-catenin by promoting the GSK-3β/β-catenin complex in HPMCs. (A) HMrSV5 cells were treated with Astragalus (800 mg/mL) and TGF-β1 (10 ng/mL). The cell extracts were immunoprecipitated with anti-β-catenin, and co-precipitated GSK-3β was analyzed by western blotting. The relative intensity of bound GSK-3β (% of control) is shown. (B, C and D) HMrSV5 cells were exposed to Astragalus (800 mg/mL) and TGF-β1 (10 ng/mL), lysed using the NE-PER nuclear and cytoplasmic extraction reagents to obtain nuclear and cytoplasmic proteins. Western blotting assays were used to detect cytosolic GSK-3β, phosphorylated GSK-3β (B), β-catenin and phosphorylated β-catenin (C). The expression of nuclear β-catenin is shown, and Histone H3 was used as a loading control (D). Data are expressed as the mean±SEM, *$p<0.05$ vs. control; #$p<0.05$ vs. TGF-β1 treatment.

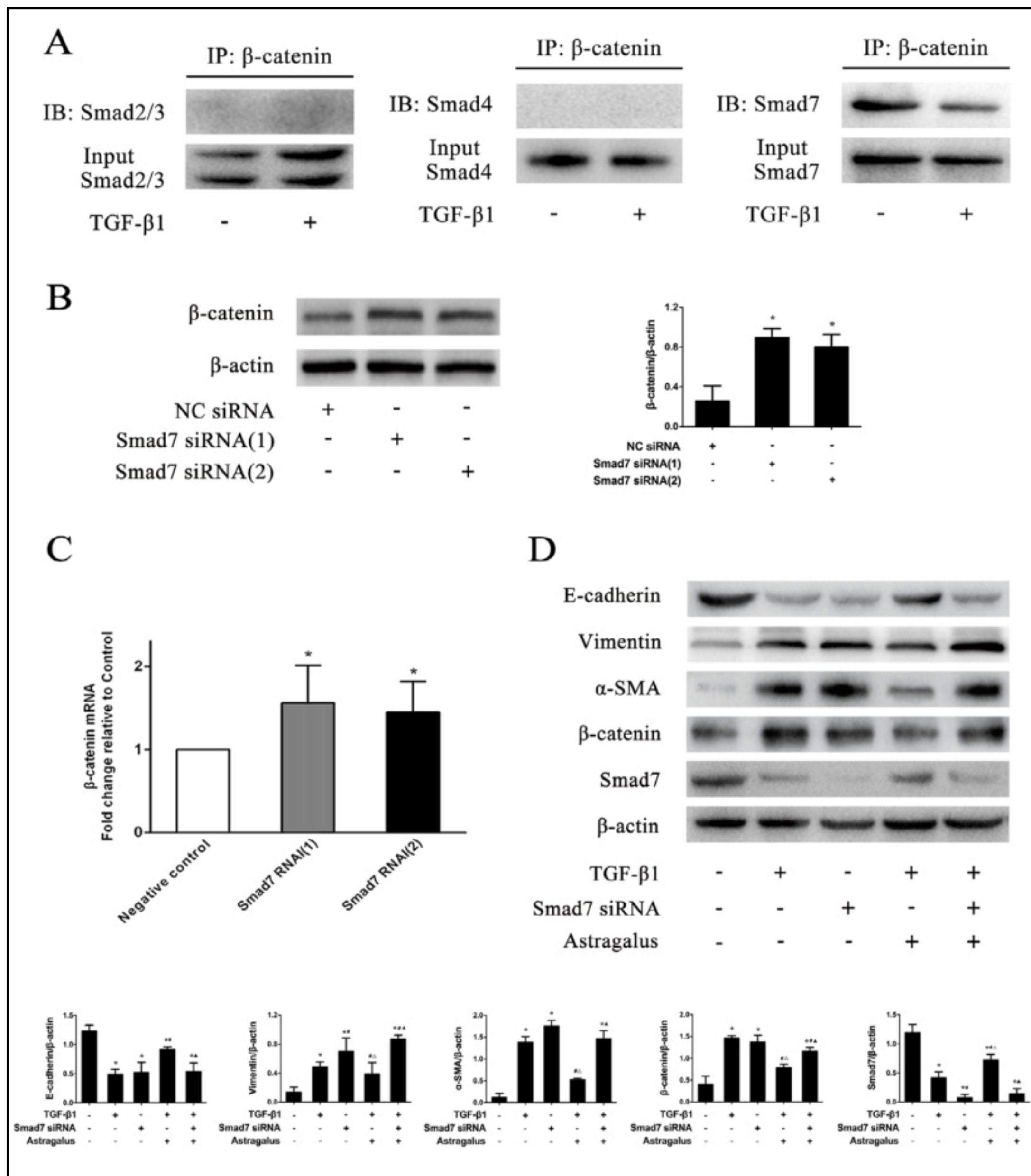

Fig. 7. Astragalus down-regulates β-catenin by enhancing the expression of Smad7 in HPMCs. (A) HMrSV5 cells were treated with TGF-β1 (10 ng/mL). The cell extracts were immunoprecipitated with anti-β-catenin, Smad2/3, Smad4 and Smad7 were co-precipitated. Western blotting was performed to analyze the expression of these proteins. (B and C) HMrSV5 cells were transiently transfected with Control Smad7 siRNA and Smad7 siRNA for 48 h, β-catenin protein and mRNA were analyzed by western blotting and real-time PCR, respectively. Data are shown as the mean±SEM, $^{*}p<0.05$ vs. NC siRNA treatment. (D) HMrSV5 cells were treated with Smad7 siRNA for 48 h and incubated with Astragalus (800 mg/mL) and TGF-β1 (10 ng/mL). Western blotting was performed to detect EMT markers, β-catenin and Smad7 expression. Data are expressed as the mean±SEM, $^{*}p<0.05$ vs. control; $^{\#}p<0.05$ vs. TGF-β1 treatment; $^{\Delta}p<0.05$ vs. Smad7 siRNA treatment; $^{\blacktriangle}p<0.05$ vs. Astragalus and TGF-β1 treatment.

Astragalus down-regulates β-catenin by enhancing the expression of Smad7 in HPMCs

TGF-β1 is well known as a vital secretory ligand that induces Smad signaling, which is the canonical pathway regulating EMT [22]. To explore the crosstalk between the Smad and

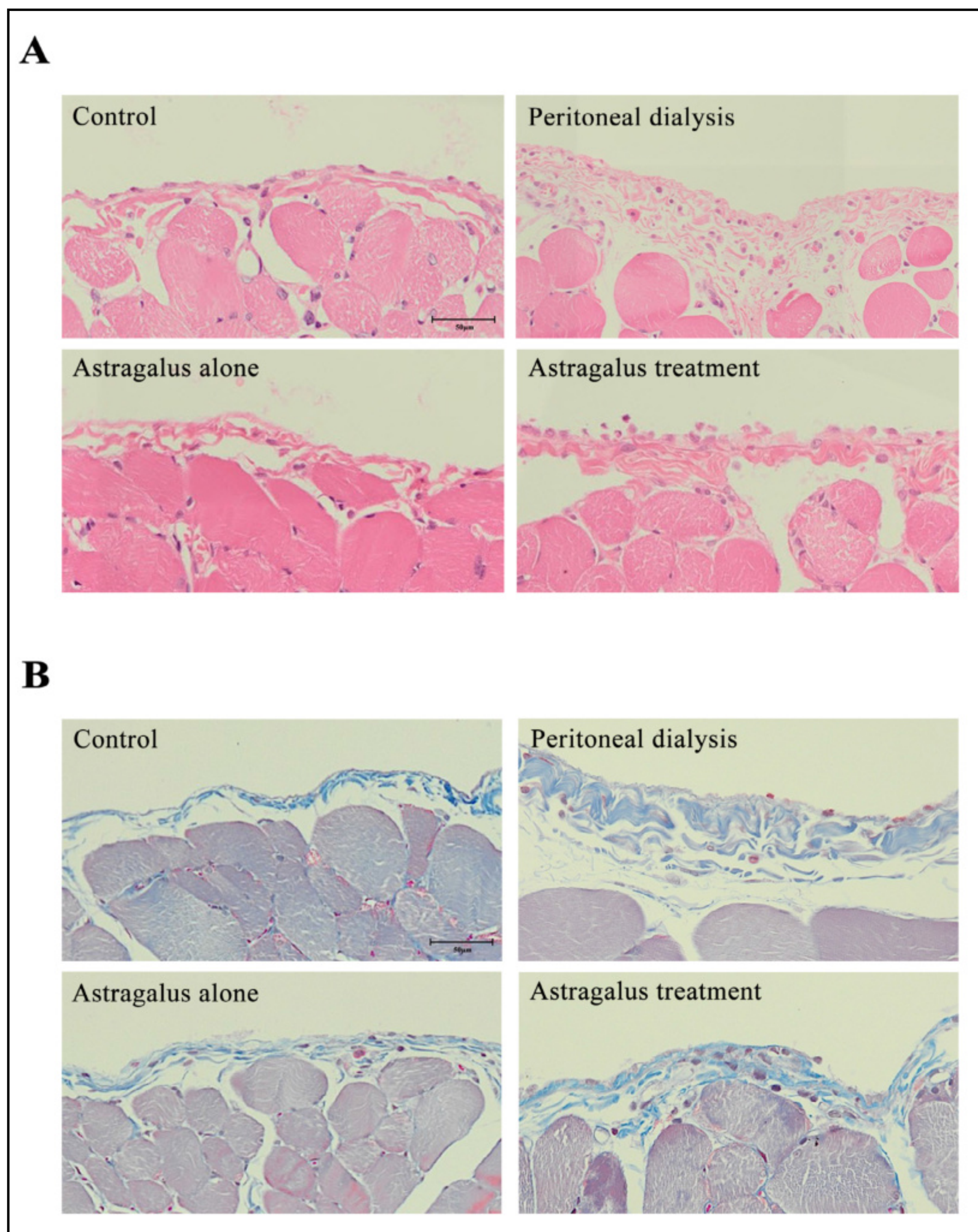

Fig. 8. Effect of Astragalus on peritoneum in PD rat. Rats were injected intraperitoneally with PD fluid, Astragalus diluted in an equal volume of saline or PD fluid. (A) HE staining of mesothelial cells of the diaphragmatic peritoneum in rats showed mesothelial injury and fibrosis (scale bar=50 μm). (B) Peritoneal thickening and ECM deposition were observed using Masson's trichrome-staining (scale bar=50 μm).

β-catenin signaling during EMT in HMrSV5 cells, we assessed binding between β-catenin and Smad proteins. Co-IP revealed that Smad7, but not Smad2/3 or Smad4, could form a complex with β-catenin in HMrSV5 cells (Fig. 7A). To further confirm the relationship between Smad7 and β-catenin, cells transfected with a Smad7 siRNA showed increased expression of β-catenin at both the mRNA and protein levels (Fig. 7B-C). Moreover, Astragalus can

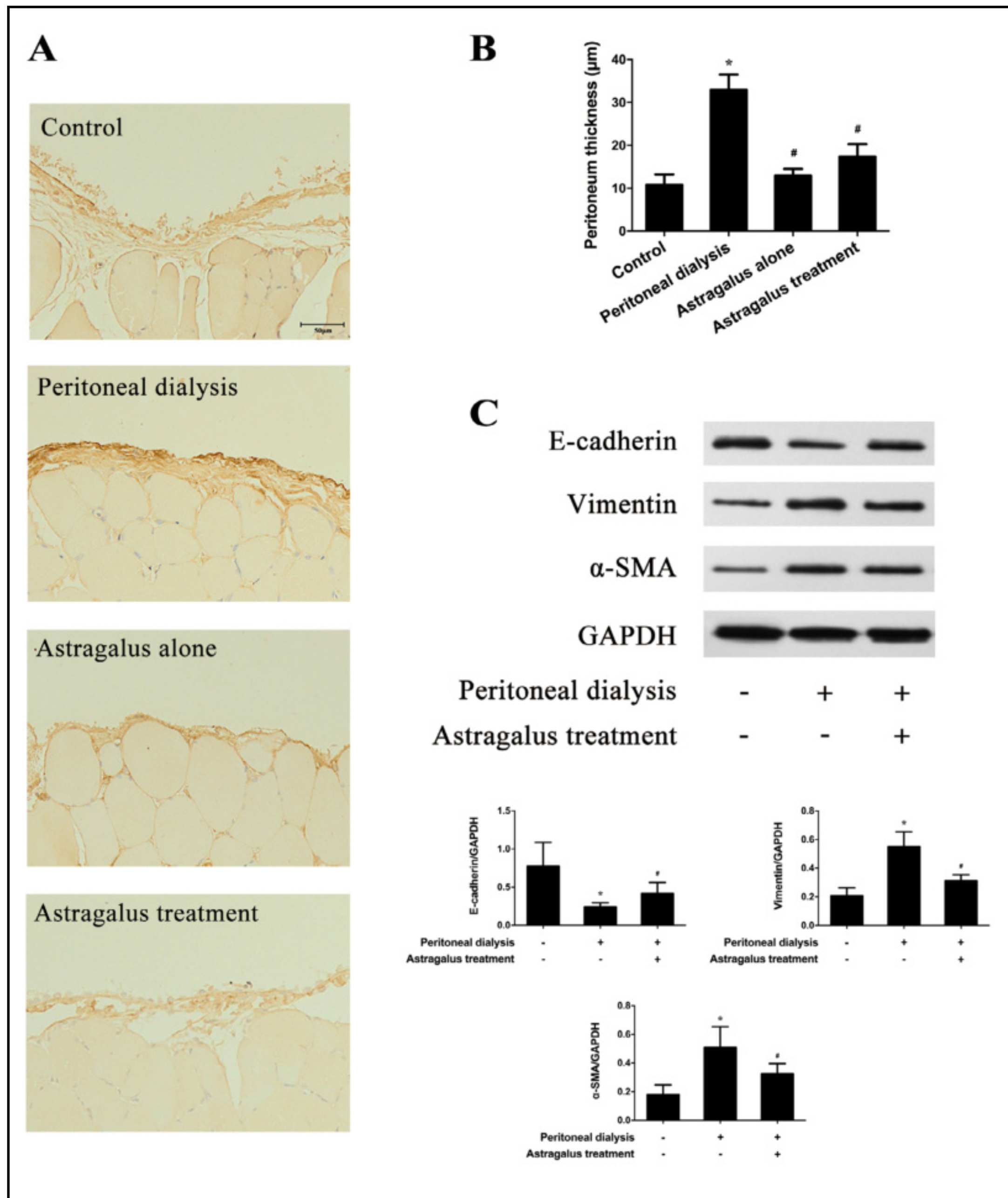

Fig. 9. Astragalus affects PF and EMT in PD rat. (A) Tissues of the diaphragmatic peritoneum were subjected to immunohistochemical staining for Collagen I expression (scale bar=50 μm). (B) The submesothelial tissue thickness was measured in the diaphragm. Data are shown as the mean±SEM. *$p<0.05$ vs. control; #$p<0.05$ vs. peritoneal dialysis treatment. (C) Tissues were homogenized using RIPA buffer for a western blotting assay. Expression of E-cadherin, Vimentin, α-SMA protein from peritoneum after PD fluid and Astragalus treatment are shown as the mean±SEM, *$p<0.05$ vs. control; #$p<0.05$ vs. peritoneal dialysis treatment.

inhibit TGF-β1-induced EMT and β-catenin expression. Smad7 knockdown down-regulated E-cadherin, whereas up-regulated Vimentin and α-SMA. Thus, Smad7 knockdown is involved in reversing the effect of Astragalus on inhibiting EMT, as well as down-regulating β-catenin (Fig. 7D). Taken together, these data suggested that Smad7 plays a crucial role in β-catenin-mediated EMT. Astragalus down-regulates β-catenin by enhancing the expression of Smad7 in HMrSV5 cells.

Astragalus attenuates PF and EMT in a rat model of PD

HE staining showed mesothelial layer denudation, submesothelial fibrosis, perivascular bleeding and inflammation in the PD rat peritoneum. Compared with the PD rats, the rats treated with Astragalus exhibited significantly alleviated mesothelial injury and fibrosis of the submesothelial zone (Fig. 8A). Masson's trichrome-staining analysis revealed fibrous capsule formation, collagen proliferation, and ECM deposition in the peritoneum of PD rats. A restorative effect of Astragalus on PF and the thickened peritoneum in the PD rat model was observed (Fig. 8B). IHC analysis was performed to observe the expression of Collagen I, which is a marker of fibrosis (Fig. 9A). Rats subjected to dialysis and Astragalus treatment exhibited a decrease in Collagen I in the mesothelial and submesothelial areas, suggesting that Astragalus protected the peritoneum from fibrosis. After peritoneal dialysis for 5 weeks, the peritoneum thickness was significantly higher in PD group than the control. Intraperitoneal injection with Astragalus caused a decrease in peritoneal thickness compared to PD group. The group treated with Astragalus alone exhibited no effects on the thickness of the peritoneum (Fig. 9B). In addition, EMT *in vivo* was observed using western blotting. As shown in Fig. 9C, PD fluid indeed declined E-cadherin expression in protein levels, as well as raised Vimentin and α-SMA. Astragalus attenuated PD fluid-induced EMT.

Astragalus down-regulates β-catenin by enhancing the expression of Smad7 in the rat model

To examine the inhibitory effect of Astragalus on EMT *in vivo*, we determined the expression of EMT markers in the fibrotic rat parietal peritoneum using IHC. As shown in Fig. 10A, the epithelial marker E-cadherin was down-regulated, whereas the mesenchymal markers Vimentin and α-SMA were up-regulated after intraperitoneal injection with PD fluid. Meanwhile, Astragalus down-regulated β-catenin in the PD fluid-treated rat model. To further elucidate the inhibitory effect of Smad7 on β-catenin and EMT, we generated the Smad7 gene transfection of rat using AAV in a vein injection with constructs inducing or inhibiting Smad7 expression. IHC and real-time PCR analysis demonstrated that Smad7 knockdown resulted in increased EMT in the PD fluid-treated rat model (Fig. 10A-B). The effect of Astragalus on the down-regulated expression of β-catenin was partially reversed by Smad7 knockdown in PD fluid-treated rats. In addition, the overexpression of Smad7 can decrease EMT and β-catenin in the rat model. Altogether, these results suggested that Astragalus down-regulates β-catenin by enhancing the expression of Smad7 in the rat model.

Discussion

In this study, we demonstrated that (i) Astragalus inhibits EMT in peritoneal mesothelial cells and in a rat model of PD; (ii) the beneficial effect of Astragalus on EMT and PF is attributed to the inhibition of β-catenin activation; (iii) the effect of Astragalus is dependent on increasing the GSK-3β/β-catenin complex, which further promotes the degradation of β-catenin; and (iv) Astragalus also protects the peritoneum from EMT and fibrosis by enhancing Smad7 to down-regulate β-catenin. All of these results proved that Astragalus ameliorates peritoneal EMT both *in vitro* and *in vivo*.

The long-term instillation of biologically incompatible PD fluid leads to PF. Morphologically, the fibrotic peritoneum is characterized by a detachment of the mesothelial layer, angiogenesis, a progressive thickening with ECM deposition and an increased presence of myofibroblasts. Myofibroblasts are the major cells responsible for collagen production, and some of these cells are activated during fibrotic progression [23]. EMT is the most important mechanism during the initial stage of PF, which has traditionally been considered a process that epithelial cells can fully convert into fibroblasts, and is thus seen as principal fibroblast-generating process responsible for tissue fibrosis [24]. Therefore, preventing or ameliorating EMT in PMCs may serve as a potential therapeutic strategy for delaying PF and improving the efficiency of PD. In particular, recent study has raised significant doubts

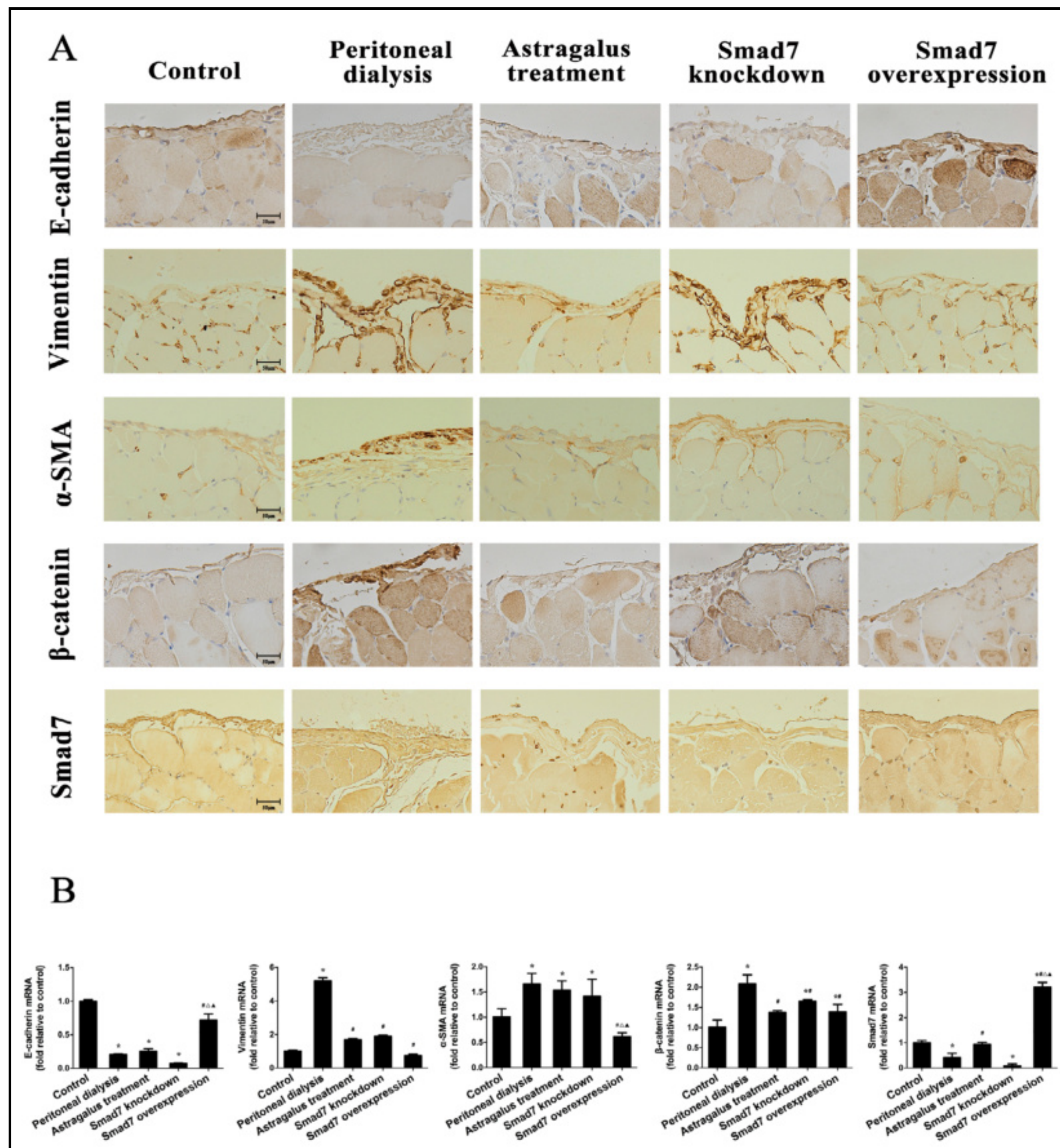

Fig. 10. Astragalus down-regulates β-catenin by enhancing the expression of Smad7 in the rat model. (A) Rats were injected once into the tail vein with AAV-Smad7 siRNA or AAV-Smad7 at a dose of 1.00E+11 v.g/mL. After 21 days, the rats were injected intraperitoneally with Astragalus or PD fluid for another 35 days. Control: normal control; Peritoneal dialysis: standard PD fluid (100 mL/kg/day); Astragalus treatment: PD fluid (100 mL/kg/day) + Astragalus (4,000 mg/kg/day); Smad7 knockdown: PD fluid (100 mL/kg/day) + Astragalus (4,000 mg/kg/day) + AAV-Smad7 siRNA (1.00E+11 v.g/mL); Smad7 overexpression: PD fluid (100 mL/kg/day) + AAV-Smad7 (1.00E+11 v.g/mL). Immunohistochemical staining of E-cadherin, Vimentin, α-SMA, β-catenin and Smad7 are shown (scale bar=50 μm). (B) Tissues were lysed using the TRIzol reagent for a real-time PCR assay. Expression of E-cadherin, Vimentin, α-SMA, β-catenin and Smad7 mRNA from peritoneum after various treatments are shown as the mean±SEM. $^{*}p<0.05$ vs. control; $^{\#}p<0.05$ vs. peritoneal dialysis treatment; $^{\triangle}p<0.05$ vs. Astragalus treatment; $^{\blacktriangle}p<0.05$ vs. Smad7 knockdown.

on the paradigm of EMT in fibrotic diseases, highlighting the role of EMT in the generation of fibroblasts/myofibroblasts [23]. Selective cell isolation and lineage-tracing experiments have suggested that the progenitor cells in the peritoneum are able to switch to fibroblast-like cells when stimulated by injury. A portion of interstitial myofibroblasts arise from PMCs

through EMT, but partial PMCs participates in PF without EMT [25]. As few studies directly contradict EMT as an origin of myofibroblasts in the context of PD, convinced studies would be welcome. In this study, we established a rat model of PD by daily intraperitoneal injection with standard PD fluid. After PD treatment, the pathological changes of the rat peritoneum showed extensive mesothelial denudation, interstitial fibrosis and a thickened submesothelial cell layer. Astragalus treatment could inhibit PF, including partially attenuating peritoneal thickening, collagen deposition, vascular proliferation. TGF-β1 is recognized as strong EMT/fibrotic inducer. Interestingly, it's a pleiotropic secreted cytokine with anti-inflammatory and immunoregulatory properties, but the underlying functional mechanisms remain unclear [26]. Several studies even indicated that TGF-β1 has opposite effect on regulating inflammation, depending on different cell cycle distribution, types, differentiation, and activation. TGF-β1 may exert chemotaxis with monocyte to recruit inflammatory cells, but also as the cytokine induced neutrophil chemoattractant to induce immune escape [27]. Here, we found Astragalus indeed inhibits inflammation in peritoneum, as well as has a dominant effect on anti-TGF-β1. It could suppress advanced glycation end products (AGE)-induced inflammatory cytokine and hence inhibit macrophage-regulated inflammation via p38 MAPK and NF-κB signaling [28]. Our previous finding, likewise, showed that the expression levels of monocyte chemoattractant protein (MCP)-1 and F4/80 (macrophage/monocyte marker) in PD rats' peritoneum were reduced after Astragalus treatment [18].

Accumulated evidence indicated that canonical Wnt/β-catenin signaling is implicated in the induction of myofibroblast proliferation [9]. β-catenin is closely related to EMT, targeting E-cadherin, Vimentin, Snail, Slug and other proteins by translocating to nucleus and binding with TCF/LEF. For example, Snail is a key transcription factor that drives EMT, which suppresses E-cadherin expression and disrupts cell-cell adhesion during early EMT [29]. Activated β-catenin signaling affects Snail as a transcriptional target and is also regulated post-translationally by GSK-3β [30]. All of these signaling factors have synergistic effects in EMT. Thus, blocking the nuclear translocation and expression of β-catenin is beneficial for antagonizing EMT. In this study, TGF-β1 induced EMT, characterized by decreasing levels of the epithelial marker E-cadherin but increasing levels of the mesenchymal markers Vimentin and α-SMA. Our results indicated that Astragalus suppresses EMT by down-regulating β-catenin. β-catenin knockdown inhibits EMT of HMrSV5, suggesting that β-catenin plays a key role in EMT inhibition by Astragalus in HPMCs.

Importantly, TGF-β1-induced EMT was accompanied by the activation of β-catenin, subsequent stimulation in the cytoplasm and nuclear translocation. It was already known that in canonical signaling, Wnt induce changes in the so-called "destruction complex", comprising Dsh, Axin, Adenomatous polyposis coli (APC), Casein kinase 1 and GSK-3β. In normal, quiescent state, β-catenin is constitutively phosphorylated by GSK-3β due to complex formation in the cytoplasm and undergoes ubiquitin-mediated proteolytic degradation. However, when Wnt engages with its ligand, it causes the stabilization and activation of β-catenin due to the phosphorylation of GSK-3β at a threonine residue. This phosphorylation allows β-catenin to translocate to the nucleus, wherein it binds to TCF/LEF to stimulate the transcription of downstream target genes, such as Snail and Slug [31]. Meanwhile, the activation of the Wnt/β-catenin pathway is required for tumor migration and invasion, leading to reduced E-cadherin, which induces EMT [32]. In the present study, we used Co-IP to confirm a role of TGF-β1-induced activation of the β-catenin signaling, which manifested as decreased β-catenin combined with GSK-3β, which was blocked by Astragalus. Western blotting showed that Astragalus down-regulates phosphorylated GSK-3β to increase the degradation of β-catenin and the amount of phosphorylated β-catenin in the cytoplasm and decrease nuclear β-catenin expression.

Smad7 is an inhibitory Smad that antagonizes the TGF-β signaling in a negative feedback loop [33]. Smad7 can recruit E3 ubiquitin ligases, resulting in the degradation of receptors and the inactivation of Smad2/3 to inhibit downstream signals of TGF-β1 [34]. Previously, we demonstrated that Astragaloside IV effectively promotes the up-regulation of Smad7 in the TGF-β1/Smad pathway during PMCs EMT. However, Smad7 can also be activated

independently of TGF-β via other factors or microenvironments. It has been demonstrated that the crosstalk between the TGF-β1/Smad and β-catenin signaling permits the tight control of critical developmental processes [35]. A previous study reported that endogenous Smad7 binds β-catenin and induces β-catenin degradation by forming a Smad7/β-catenin complex [36]. Smad7 protected β-catenin from phosphorylation and degradation by recruiting Smurf2 and E3 ligase [37]. Consistent with this concept, our study showed that Smad7 can stabilize β-catenin in PMCs to mediate EMT. β-catenin was found by Co-IP to combine with Smad7 rather than Smad2/3 or Smad4 after TGF-β1 treatment, suggesting that Smad7 is involved in β-catenin degradation and regulates the assembly of β-catenin. To further confirm the role of Smad7 in the inhibitory effect of Astragalus on β-catenin and EMT, we generated Smad7 knockdown cell lines. The knockdown of Smad7 in HMrSV5 cells reversed the inhibitory effect of Astragalus on increased β-catenin and TGF-β1-induced EMT. On the other hand, Smad7 knockdown up-regulated β-catenin by western blotting and real-time PCR assays. *In vivo*, we established Smad7 gene transfection of rats, including the overexpression Smad7 and a Smad7 knockdown rat model. IHC and real-time PCR analysis revealed that enhanced Smad7 expression leads to decreased expression of β-catenin in response to standard PD fluid. The suppressive effect of Astragalus on PD fluid-induced EMT and activated β-catenin were restored by Smad7 silencing. Therefore, we confirmed that the inhibitory activity of Smad7 serves as a crucial mediator of β-catenin expression. Astragalus down-regulates β-catenin by enhancing Smad7 and complex formation by these proteins, further improving the degradation of β-catenin.

In this study, the most important finding is a validation of the antifibrotic effect of Astragalus in the peritoneal mesothelium through the regulation of β-catenin activation. Astragalus down-regulates β-catenin by stabilizing its complex with GSK-3β and promoting Smad7. These mechanisms highlight the role of Astragalus in the regulatory effect of β-catenin and its vital function during PMCs EMT.

Conclusion

In summary, our study illustrated the inhibitory effect of Astragalus on β-catenin and EMT *in vitro* and *in vivo*. The effect of Astragalus on peritoneal EMT was demonstrated to be regulated by β-catenin signaling. Astragalus down-regulates β-catenin by promoting the GSK-3β/β-catenin complex and enhancing Smad7 expression. Our findings reveal a potential therapeutic use of Astragalus in the prevention or treatment of PF.

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Disclosure Statement

All authors have no conflicts of interest to declare.

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