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Original Article

Anti-fibrosis and relative mechanism of salvianolic acid A on rat model with renal fibrosis

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Abstract: Chronic kidney disease, as a global health problem, has gained remarkable increase and significant attention in the recent decades. It has been demonstrated that TGF-β1/Smad signaling pathway plays a critical role during the progression in CKD. Salvianolic acid A is the active component of *Salviae Miltiorrhizae* Bunge, and has been reported to have various pharmacological activities for hepatic, pulmonary and cardiac fibrosis. In the present study, with rat model, we demonstrated that salvianolic acid A is capable to repress renal fibrosis, help improve renal function by inhibiting the TGF-β1/Smad signaling pathway and display similar effect as Cozaar.

Keywords: Salvianolic acid A, renal fibrosis, TGF-β1/smad signaling pathway, traditional Chinese medicine

Introduction

Chronic kidney disease (CKD) is a major public health problem globally. In the past decades, CKD has gained a significant attention and its incidence has increased remarkably [1]. Current studies demonstrated that people with CKD would be exposed at higher risk for cardiovascular and all-cause mortality [2, 3]. Although great efforts have been invested in exploring therapies for CKD, the number of CKD patients requiring dialysis and kidney replacement keeps going up [4]. Moreover, the currently applied treatment modalities and donor kidney availability are insufficient, which further increase the demand for new approaches for chronic nephropathy treatment.

Several factors have been identified to be involved in the onset and progression of CKD [5, 6]. The process of renal tubulointerstitial fibrosis is characterized by extracellular matrix deposition, interstitial myofibroblast proliferation and the infiltration of inflammatory mononuclear cells, which are thought to play an important role in the pathogenesis of CKD [7]. The role of transforming growth factor beta (TGF-β) as a major profibrotic cytokine in various fibrotic diseases in multiple organ systems and in particular in experimental renal disease has been well studied [8]. It has been demonstrated that TGF-β1/Smad signaling pathway plays a critical role during the progression in CKD [9].

Treatment with traditional Chinese medicine might slower the progression of CKD [10]. But it is hard to shed light on the mechanism. Salvianolic acid A (SAA) is the active component of *Salviae Miltiorrhizae* Bunge. Previous studies have demonstrated that SAA has various pharmacological activities and has been widely used in the therapies for hepatic [11], pulmonary [12] and cardiac fibrosis [13], as well as nephropathy [14]. The present study aimed to evaluate both the *in vitro* and *in vivo* activity of SAA on renal fibrosis and explore whether it can help improve the condition of renal fibrosis by inhibiting TGF-β1/Smad signaling pathway.

Materials and methods

Cells and animals

Normal rat kidney interstitial fibroblast cells (NRK-49F) and Rat HBZY-1 cells line purchased

Table 1. Primers for real-time quantitative PCR used in this study

Name	Sequence (5'-3')	Target gene
TB1-F	GAAGGACCTGGGTTGGAAGT	TGF-β1
TB1-R	CGGGTTGTGTTGGTTGTAGAG	
S3-F	GGAGGAGGTGGAGAA	Smad3
S3-R	CACACTCGCTTGCTC	
C1-F	CGTGGAAACCTGATGTATGCT	Collagen1
C1-R	ACTCCTATGACTTCTGCGTCTG	
FN-F	AAGGCAATGGGCGTATCAC	FN
FN-R	TGGGTCTGGGGTTGGTAAAT	
BA-F	CCTCTATGCCAACACAGT	β-actin
BA-R	AGCCACCAATCCACACAG	

from Shanghai Fymen Gene Biological Technology Company. Adult male Sprague-Dawley rats weighing 180-200 g were supplied by Shanghai Super-B&K Laboratory Animal Company. Rats were taken into a special room with stable ambient temperature of 18-22°C and housed in wire cages with free access to standard diet and tap water. Experiments were performed according to the institutional guidelines and were approved by the Shanghai University of Traditional Chinese Medicine.

Chinese herb monomer SAA was purchased from Shanghai Winherb Medical Technology Company ($C_{26}H_{22}O_{10}$, MW 494.45, Purity 99%). Cozaar was purchased from Hangzhou MSD Pharmaceutical Company.

Cell culture, grouping and detection

NRK-49F and HBZY-1 cells were digested in trypsin-EDTA solution (0.25% trypsin and 0.02% EDTA) and cultured in DMEM/F12 (containing 100 U/ml penicillin, 100 mg/ml streptomycin and 10% FCS). When achieved 70-80% confluence, cells were plated (1×10^4 cells/well) in 96-well plates, each pore 100 μl. After cells adhering to the wall, serum-free medium were applied and placed in 37°C with 5% CO_2. Cells were grown for 24 h. Experiments were conducted in 5 different mediums: blank group (DMEM medium only), TGF-β1 group (DMEM medium with 2 ng/ml TGF-β1), SAA group (DMEM medium with 49.4 μg/ml SAA), and TGF-β1 + SAA group (DMEM medium with 2 ng/ml TGF-β1 and 49.4 μg/ml SAA). MTT assay was used to examine the effect of SAA on proliferation of NRK-49F and HBZY-1 cells that were induced by TGF-β1. Six samples were used for each group, and each experiment was repeated in triplets.

Animal model treatment

5/6 nephrectomy rat model was made by Platt subtotal nephrectomy. Surgical procedures were performed under general anesthesia. Briefly, after an abdominal incision, the left kidney was exposed and separated from the adrenal gland. The lower and upper poles were tied for the resection of two thirds of the left kidney. After one week, the right kidney was removed. The sham group rats underwent the same abdominal incision and manipulation of the left and the right kidneys without tissue destruction.

40 rats were randomly divided into sham group (n=10) and surgery group. After the surgery ended, the rats in surgery group were divided into Model group (n=10), Cozaar group (n=10), and SAA group (n=10). There was no significant difference between these two groups on serum creatinine (Scr) level. Cozaar and SAA groups were gavaged with drugs of 8.6 mg/(kg.d), 17.1 mg/(kg.d), respectively. Surgery groups and sham groups were given the same amount of distilled water. After 8 weeks of treatment, rats were anesthetized with chloralhydrate (400 mg/kg) by intraperitoneal injection and blood was drawn from the abdominal aorta. Blood urea nitrogen (BUN), serum creatinine (Scr) and proteinuria were examined. Rats were housed in metabolic cages separately 24 h for urinary protein detection. All parameters were detected on the automatic biochemistry analyzer according to the user manual. At the end of the study, the rats were killed and kidney samples were harvested. Central slide from kidney was fixed in formalin and embedded in paraffin by routine methods. The remaining tissue was snap-frozen and stored at -80°C until used for detecting the levels of TGF-β1, Smad3, Collagen1, FN protein and mRNA expression on rat renal tissue by Western blot and real-time PCR method.

Western blot analysis

Protein was extracted using the RIPA buffer containing cocktail proteinase inhibitors (Thermo Fisher Scientific Inc., Rockford, IL) and

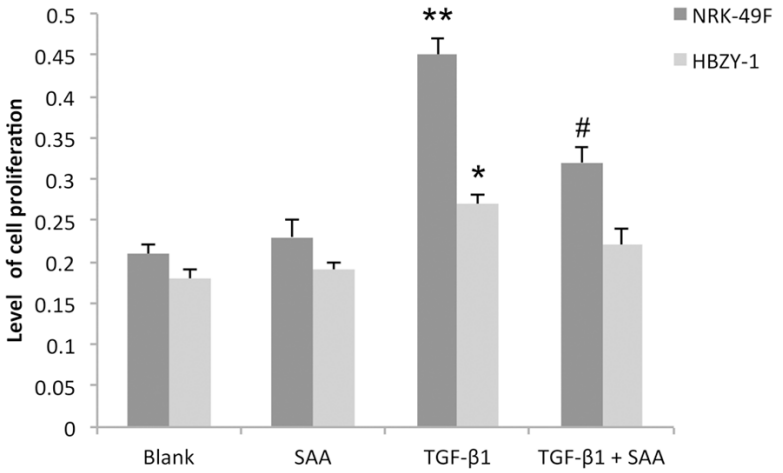

Figure 1. Effect of SAA on the proliferation of NRK-49F and HBZY-1 cells after 24 h induction by TGF-β1. Data are presented in Mean ± SD. ** and * indicate P<0.01 and P<0.05 in student t test with comparison to SAA group, respectively. # indicates P<0.05 in student t test with comparison to TGF-β1 group.

quantified with Bio-Rad protein assay. Equal amount of protein was separated on SDS-polyacrylamide gels in a Tris/glycine buffer system, transferred onto nitrocellulose membranes, and blotted according to standard procedures with primary antibodies overnight followed by incubation with appropriate fluorescence-conjugated secondary antibodies. The proteins of interest were analyzed using an Odyssey IR scanner, and signal intensities were quantified using NIH Image/J software. Anti-β-actin antibody was used as an internal control. All antibodies purchased from Cell Signaling Technology.

Real time quantitative PCR

Target mRNA expression evaluation was performed with real time quantitative polymerase chain reaction (qRT-PCR) with the relative standard curve method. Total RNA was extracted from snap-frozen kidney tissues with TRIzol Reagent (Invitrogen). Total RNA were reverse-transcribed and amplified in triplicate using IQ SYBR green supermixreagent (Bio-Rad, Herculus, CA) with a real-time PCR machine (Bio-Rad, Herculus, CA), according to the instruction. Quantitation of the relative expression was normalized to the β-actin, using the ΔΔCT method. All experiments had at least biological duplicates and assay triplicates. The specificity of real-time PCR was confirmed by melting curve analysis. Primer sequences are shown in **Table 1**.

Statistical analysis

Data were presented in mean ± standard deviation (SD). Statistical analysis was performed using SPSS 13.0 software (SPSS Inc. Chicago, IL). Analysis of variance (ANOVA) with the post-hoc Bonferroni adjustment was used for comparisons between multiple groups. In cases where there were only two groups for comparison, the two-tailed student's t test was used. Difference with *P* value <0.05 was considered as statistically significant.

Results

Effect of SAA on the proliferation in NRK-49F and HBZY-1 cells induced by TGF-β1 for 24 h

Four treatments were used to investigate the effect of SAA on cell proliferation. The SAA alone had no significant effect on the proliferation of both NRK-49F and HBZY-1 cells (P>0.05), while the use of TGF-β1 alone could promote the cell proliferation (P<0.05) (**Figure 1**).

Compared to the solo use of TGF-β1, TGF-β1 + SAA could significantly inhibit proliferation and cellular viability of NRK-49F (P<0.05), but not of HBZY-1. From this result, it could be inferred that SAA can suppress the proliferation of NRK-49F cells induced by TGF-β1. In HBZY-1 cells, though suppression could be observed, it is not statistically significant (**Figure 1**).

Effect of SAA on histological changes

In rats of Sham group, the glomerular extracellular matrix and mesangial cells presented normal distribution. Capillary lumens opened well. The kidney tubules and interstitial region were clear in structure. The epithelial cells in the kidney tubule were square, aligned well with the same size. No fibrous tissue proliferation presented in mesenchyme. In contrast, the rats in model group showed obvious congested and swollen glomeruli. The renal capsule was narrow. The tubule epithelial cells were disintegrated, denatured and dropped off. The tubule lumens were dilated containing albumen and

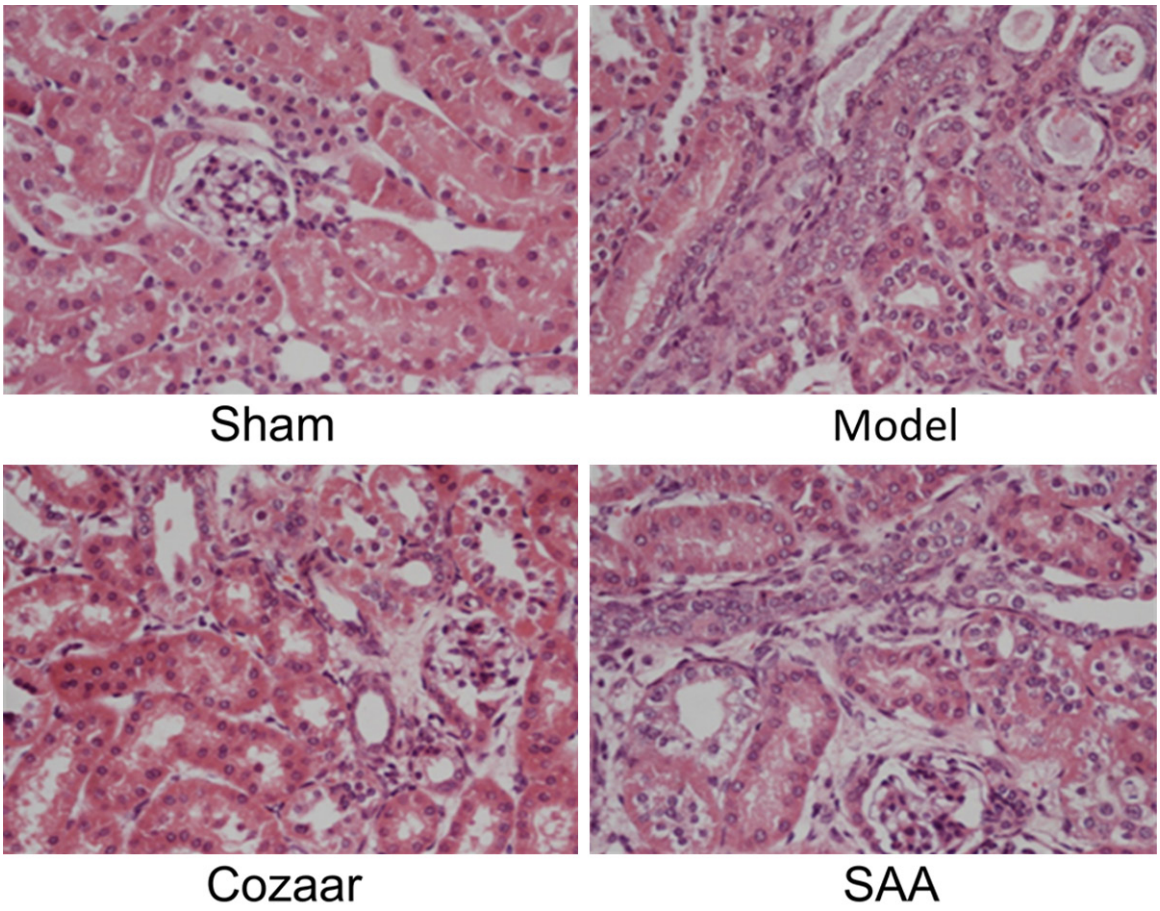

Figure 2. Effect of SAA on histological changes. Sections of the rat kidney were stained with HE. Magnifications were 400× in all images.

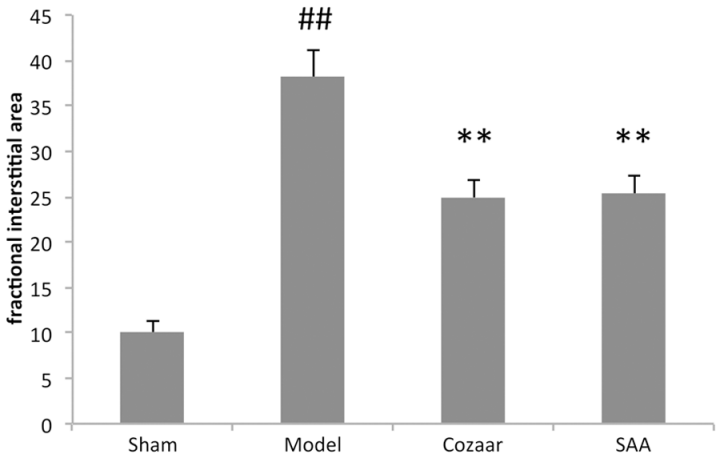

Figure 3. The fractional interstitial area in each group. Data are presented in Mean ± SD. ** indicates P<0.01 in student t test with comparison to model group. ## indicates P<0.01 in student t test with comparison to sham group.

erythrocyte cast. The tubule walls were stripped and cracked. The interstitial tissue of proximal convoluted tubule presented remarkable fibrosis. Such pathological changes were slighter in the rats of Cozaar and SAA groups (**Figure 2**). The ratio of the positive area was ameliorated after treatment with Cozaar and SAA, as indicated by reduced tubulointerstitial injury and interstitial fibrosis expansion. There was no difference between the Cozaar and SAA groups (**Figure 3**).

The effects of SAA on BUN, Scr and proteinuria

The levels of BUN, Scr and proteinuria increased in model group compared to the sham group, and decreased significantly in the Cozaar and SAA group (P<0.05). For the two treated groups, levels of BUN and proteinuria of rats in SAA group were significantly higher than those in Cozaar group, as shown in **Table 2**.

Table 2. Levels of BUN, Scr, and proteinuria in each group

Group	BUN (mg/dl)[1]	Scr (mg/dl)[1]	Proteinuria (mg/24 h)[1]
Sham	40.34±3.12	0.29±0.03	4.06±0.27
Model	95.72±6.61##	0.68±0.05##	11.45±1.00##
Cozaar	77.13±4.98§	0.59±0.02§	8.62±0.48§
SAA	69.18±3.92§,*	0.54±0.04§,*	8.07±0.39§,*

[1]Data are presented in Mean ± SD; ##P<0.01 in student t test, compared to Sham group; §P<0.05 in student t test, compared to Model group; *P<0.05 in student t test, compared to Cozaar group.

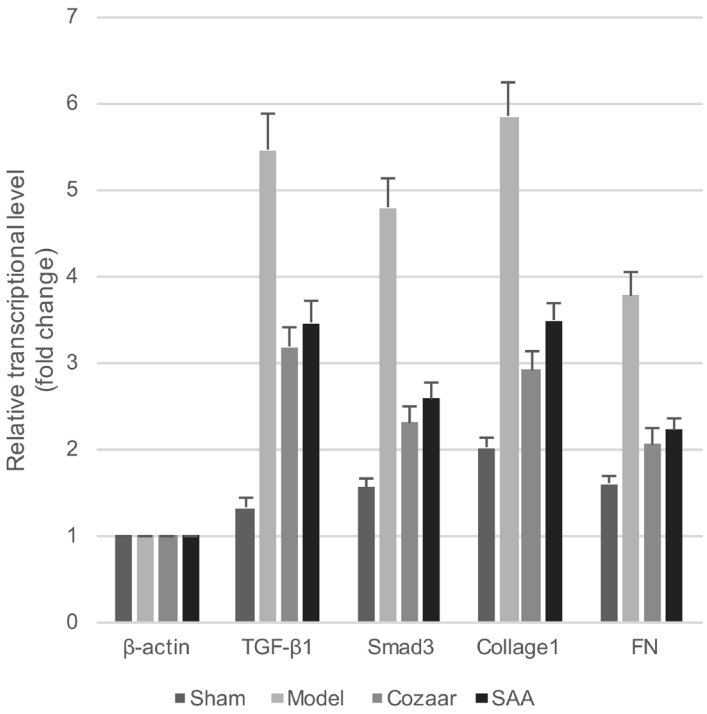

Figure 4. The effects of SAA on the levels of TGF-β1, Smad3, Collage1 and FN mRNA transcriptional in each rat renal tissue. Data are presented in Mean ± SD. β-actin gene was used as internal control. ## and # indicate P<0.01 and P<0.05 in student t test with comparison to sham group, respectively. ** indicates P<0.01 in student t test with comparison to model group.

The effects of SAA on the levels of TGF-β1, Smad3, Collage1 and FN mRNA transcription

The TGF-β1, Smad3, Collagen1 and FN mRNA transcriptional level increased in model group and decreased significantly in the Cozaar and SAA group (P<0.01 and P<0.05, respectively). For the drug treated groups, the transcriptional level of Collage1 gene was significantly higher in Cozaar group, compared to the SAA group. There are no remarkable differences for other transcripts between these two groups, as shown in **Figure 4**.

The effects of SAA on the levels of TGF-β1, Smad3, Collagen1 and FN protein expression

The TGF-β1, Smad3, Collage1 and FN protein expression increased in model group and decreased significantly in the Cozaar and SAA group (P<0.01 and P<0.05, respectively). But there was no significant difference could be observed for Smad3 between groups. There are no significant differences between Cozaar and SAA groups, for all the proteins detected. Thus SAA have the same effect as Cozaar, as shown in **Figure 5**.

Discussion

Tubulointerstitial fibrosis is the final common pathway in late-stage renal disease. The pathogenesis of kidney fibrosis is characterized by overproduction and deposition of extracellular matrix (ECM), which ultimately leads to fibrotic lesions and tissue scarring [15, 16]. Renal interstitial fibroblasts are the principal effector cells that responsible for ECM overproduction in the fibrotic kidney. Their activation is regarded as a key event in the pathogenesis of chronic renal fibrosis [17, 18]. TGF-β1 causes augmented extracellular matrix protein deposition (collagen types I, IV, V, and VI; fibronectin (FN), and laminin (LA)) at the glomerular level, thus inducing mesangial expansion and glomerular basement membrane thickening. Previous research suggested that the aberrant expression of TGF-β led to the activation of ECM synthesis and dysfunction of ECM degradation simultaneously [19], resulting in the unbalance between ECM production and degradation and thus fibrosis. During chronic renal injury, TGF-β is involved in this process causing progression of renal fibrosis. Smad2/3 proteins have been identified to have an important function in the expression of extracellular matrix (ECM) regulation through TGF-β-Smad2/3 signaling pathway. TGF-β1 is a major cytokine that induces transformation of quies-

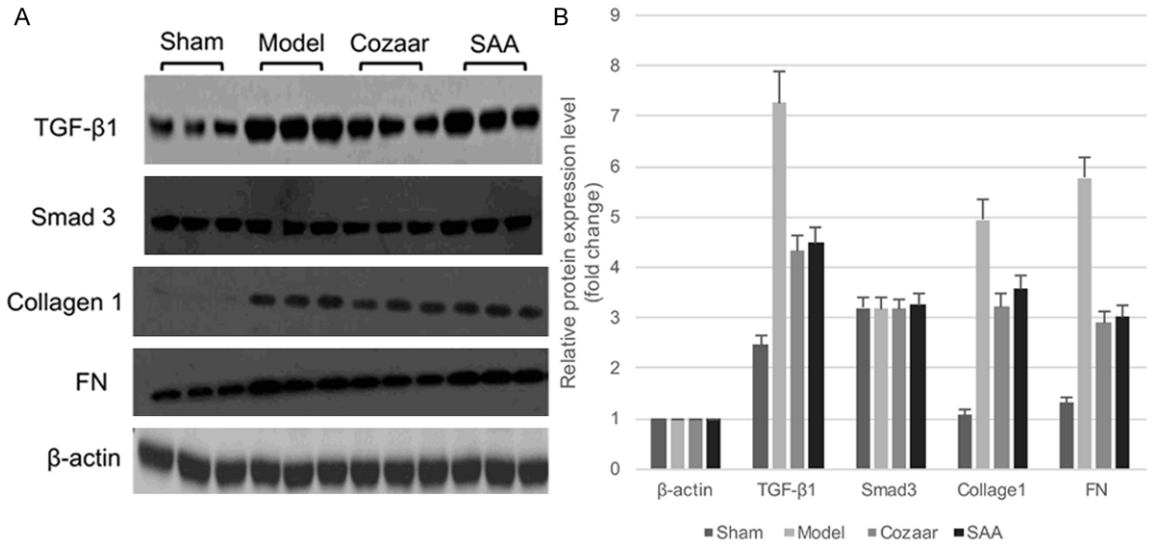

Figure 5. The effects of SAA on the levels of TGF-β1, Smad3, Collage1 and FN protein expression in each rat renal tissue. A. Semi-quantification of protein expression level by Western blotting. B. Signal intensities of Western blotting quantified by NIH Image/J software. Anti-β-actin antibody was used as an internal control. ** indicates P<0.01 in student t test with comparison to model group. ## indicates P<0.01 in student t test with comparison to sham group.

cent renal fibroblasts to myofibroblasts through activation of Smad2/3 [20, 21].

Although the mechanism of myofibroblast activation and the fibrogenesis under various pathologic conditions have not been completely understood, activation of multiple cytokines/growth factor receptors is involved in these processes [22, 23]. Injury to the kidney is associated with release of cytokines/growth factors such as TGF-β, EGF, and PDGF by damaged or infiltrating cells [24, 25]. An increase in production of TGF-β is one of the most important mechanisms in the pathogenesis of renal fibrogenesis [26]. TGF-β1 stimulates fibroblast cell activation and induces matrix expression through its interaction with TGF-β receptors, which are mainly composed of two protein families-type I (TβRI) and type II (TβRII) receptors [27], TGF-β1 binds to TβRII, which results in TβRI recruitment to form a heteromeric TGF-β receptor complex. The complex phosphorylates and activates Smad2 and Smad3, the two major Smads that mediate the profibrotic events [28].

Smad3 is a member of the receptor-activated Smads (RSmads), which are central mediators of TGF-β signaling. Induction and activation of the profibrotic cytokine TGF-β in the kidney, either alone or in combination with other cytokines such asepidermal growth factor, fibroblast growth factor-2 or angiotensin II, induces kidney damage through a range of pathobiological processes, including EMT, apoptosis and fibrosis [29]. Smad3 is also involved in other non-redundant signaling pathways and biological functions [30]. These complexes translocate to the nucleus where they control expression of target genes. CTGF is a key effector in the downstream of TGF-β and plays a role in the regulation of fibroblast proliferation and migration as well as TGF-β-dependent ECM production [31].

Currently, drug discovery efforts for fighting renal fibrosis are largely focused on compounds that are specific for a particular receptor or protein kinase. Standard therapies to delay CKD progression include dietary protein restriction and administration of angiotensin-converting enzyme inhibitors (ACEI) and angiotensin receptor blockers (ARB) to help control blood pressure and confer additional renoprotective effects. Despite such interventions, CKD incidence and mortality rates continue to increase. Given that renal fibrogenesis is associated with increased production of multiple cytokines/growth factors and subsequent activation of their receptors and signaling pathways, it is expected that inhibitors with broad specificity might offer improved therapeutic benefit in kidney fibrotic diseases. Because activation of

TGF-β signaling is considered to be the major mechanism that directly promotes fibroblast activation and fibrosis progression, therapeutic intervention of this pathway could be considered as a strategy to halt or prevent renal fibrosis.

Traditional Chinese medicines, has received increasing attention as a mean of alternative and complementary therapies, *Salvia miltiorrhiza* were used widely in China to treat CKD and has been reported to offer a range of pharmacological properties that may delay disease progression [10, 32].

SAA is the main active constituent of *Salvia miltiorrhiza*. It is extracted from the dried-root and rhizome of *Salvia miltiorrhiza* Bunge, which possesses antioxidant, anti-inflammatory, antiplatelet properties. Recently, it has been suggested that SAA displays cardioprotective effects against myocardial IRIo [33-35], its role and anti-fibrosis mechanisms have not been clearly expound, so we aimed to explain the molecular mechanisms of SAA on renal failure by regulating TGF-β1/Smad signaling activity in model rat and for the first time shown SAA have antifibrotic properties because it inhibited of TGF-β1/Smad and has slowed the progression of kidney disease in 5/6 nephrectomy rat model.

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Disclosure of conflict of interest

None.

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