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*Original Research*

# Bone marrow mesenchymal stem cell-derived exosomes improve renal fibrosis via regulating Smurf 2/Smad 7

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**Abstract**

**Background**: Exosomes can be secreted from bone marrow mesenchymal stem cells (BMSCs) to extracellular space and exert anti-fibrotic effects, but the underlying mechanisms remain to be elucidated. **Methods**: 5/6 subtotal nephrotomy (SNx) rat models and TGF-$\beta$1-induced human renal proximal tubular epithelial cells (HRPTEpiCs) were established to simulate renal fibrosis. Renal function and fibrosis were assessed by Hematoxylin and Eeosin (HE) staining, Masson staining, immunohistochemistry, and western blot. The expression of Smad 7/Smurf 2 was detected in rats and HRPTEpiCs by western blot, and a further potential mechanism was explored using si-Smurf 2. **Results**: BMSC-Exo improved renal function, reduced the fibrotic region, down-regulated the expression of fibronectin, Collagen-I, $\alpha$-SMA, and up-regulated E-cadherin in SNx models. *In vitro* study demonstrated that knocking down the expression of Smurf 2 significantly increased the expression of Smad 7, which could be enhanced by BMSC-Exo. BMSC-Exo could alleviate the fibrosis induced by TGF-$\beta$1 in tubular epithelial cells and enhanced the protective effect of si-Smurf 2 on renal fibrosis. **Conclusions**: BMSC-Exo inhibited renal fibrosis both *in vivo* and *in vitro*, partially, by regulating the Smurf 2/Smad 7 axis. BMSC-Exo enhanced the protective effect of si-Smurf 2 on fibrosis induced by transforming growth factor-$\beta$1 (TGF-$\beta$1).

**Keywords:** Renal fibrosis; Bone marrow mesenchymal stem cell; Exosome; Chronic kidney disease; Smurf 2/Smad 7

## 1. Introduction

Chronic kidney disease (CKD) is a global health issue that leads to lower quality of life and higher social economic burden [1], especially for patients who need renal replacement therapy. The advent of dialysis and kidney transplantation improved the survival rate and quality of life for patients with End-stage renal disease (ESRD), however, both of the two treatments were costly and with multiple complications [2,3]. Thus, effective and affordable treatments need to be developed to delay or reverse the progression of CKD. Renal fibrosis is a pathological process and a common final pathway which makes progression to ESRD [4]. Renal fibrosis is characterized by the imbalanced deposition/degradation of extracellular matrix (ECM) [5]. Transforming growth factor-$\beta$1 (TGF-$\beta$1) plays critical roles in renal fibrosis via canonical (Smad-based) and non-canonical signaling pathways [6]. The Smad subfamily has been classified as receptor Smad (R-Smad), common mediator Smad (Co-Smad) and inhibitory Smad (I-Smad), among which Smad 7 acts as a key antagonist of TGF-$\beta$ signaling pathway thereby mediating the R-Smad dephosphorylation or degradation [7]. The anti-fibrotic effect of Smad 7 has been demonstrated in different kidney disease models of animals such as diabetic nephropathy, obstructive nephropathy, and aristolochic acid nephropathy [8]. The E3 ubiquitin ligase Smad ubiquitination regulatory factor 2 (Smurf 2) can specifically target components of TGF-$\beta$ signaling family which lead to the ubiquitination and degradation of smad 7 to deteriorate kidney fibrosis. Previous studies indicated that the upregulation of Smurf 2 accompanied with downregulation of R-Smads subfamily, which denoted the activation of TGF-$\beta$ signaling. For example, Smurf 2-transgenic mice sternal chondrocytes showed an increased Smad 3 degradation [9]. Similarly, the increased expression of Smurf 2 correlates with decreased Smad 2 expression in rat glomeruli [10]. However, Smurf 2 also exhibited an effect in facilitating TGF-$\beta$ response by degrading Smad 7 through a post-transcriptional mechanism. The role of Smurf 2 under different pathophysiology context remains complicated and need to be further clarified.

Mesenchymal stem cells (MSCs) are a type of multipotent stem cells derived from mesoderm and ectoderm which are considered to be a potential therapy in organ injury, repair and immune response. Liu *et al.* [11] reported that BMSCs function as inhibitory role of renal inflammation and fibrosis after injury. Moreover, Ozbek *et al.* [12] found that MSCs delivery could significantly alleviate renal fibrosis via inhibiting epithelial-mesenchymal transition in unilateral ureteral obstruction (UUO) rats. Although the exact way of BMSCs on restoring kidney injury remains

ambiguous, substantial results demonstrated that endocrine and paracrine mechanisms are of vital importance in this progression [13,14].

Exosomes are 30–100 nm nanoscale lipid bilayer vesicles that can be secreted from different cells (such as bone marrow mesenchymal stem cells, renal tubular cells, macrophages, cancer cells) to extracellular space and exert anti-inflammatory and anti-fibrotic effects [15]. Several studies demonstrated that BMSC-derived exosome could reduce inflammation and extracellular matrix deposition in hepatic fibrosis rats via inhibiting TGF-$\beta$/Smad 2 signaling pathway [16]. Furthermore, the infiltration of dendritic cells could be significantly suppressed by MSC-derived exosome in kidney as well as the expression of inflammatory cytokines in streptozotocin (STZ)-induced diabetic mice [17]. Up to now, the underlying mechanism of the anti-fibrotic effect of exosomes still needs further elucidation.

Our current study aims to explore whether BMSC-Exo exerts the anti-fibrosis effects on a 5/6 nephrectomy model and TGF-$\beta$1-induced tubular epithelial cells. In addition, the underlying mechanism of BMSC-Exo mediated anti-fibrosis effects were investigated and we confirmed that Smurf 2/Smad 7 axis played a vital role in this progression. Our findings not only lead to a better understanding of the protective role of BMSC-Exo in fibrosis but also provides a potential therapeutic strategy for CKD.

## 2. Materials and methods

### *2.1 Isolation of BMSC-Exo*

Human bone marrow mesenchymal stem cells (BMSCs) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured in MSC medium supplemented with 10% exosome-depleted FBS (Thermo Fisher, cat. A25904DG, Waltham, MA, USA). Primary rat bone marrow mesenchymal stem cells were obtained from rat femur and cultured in DMEM medium supplemented with 10% exosome-depleted FBS. The differentiation potential of bone marrow mesenchymal stem cells were identified using alizarin red S staining and oil red O staining. After 72 h, the conditioned medium was collected and centrifuged (1000 $\times$ g for 10 min, 9000 $\times$ g for 30 min) at 4 °C to remove cell debris, followed by centrifugation at 3000 $\times$ g for 90 min (4 °C) after moving into the ultrafiltration centrifuge tube. Then Exosome Isolation Reagent (Invitrogen, Carlsbad, CA, USA) was used in accordance with the manufacturer's protocol to isolate exosomes. The exosome-enriched fraction was diluted with PBS and quantified using a Pierce BCA protein assay kit before further use. BMSCs used in our study is from 4–6 passages.

### *2.2 Nanoparticle tracking analysis (NTA) and transmission electron microscopy*

Exosome-enriched suspensions were examined by the ZetaView Particle Metrix (PMX 110, Particle Metrix, Meerbusch, Germany), and particle movement was analyzed using NTA software ZetaView 8.02.28 (Particle Metrix, Meerbusch, Germany). The exosome-enriched solution was placed on a copper mesh and stained with a uranyl acetate solution finally photographed by a Transmission electron microscope (TEM, Hitachi, Tokyo, Japan).

### *2.3 Cell culture and treatment of HRPTEpics*

Human renal proximal tubular epithelial cells (HRPTEpiC) were purchased from ScienCell Research Laboratories and cultured in HRPTEpics medium supplemented with 5% FBS. TGF-$\beta$1 (10 ng/mL; 7754-BH-025/CF, R&D Systems, USA) was used to induce fibrosis. The siRNA targeting Smurf 2 (si-Smurf 2) was purchased from Genechem (Shanghai, China) and transfected into HRPTEpics using the transfection reagent according to the manufacturer's protocol. Human BMSC-Exo was used to stimulate HRPTEpiC.

### *2.4 Animal models*

Briefly, 12-week-old male Sprague-Dawley rats (200 $\pm$ 20 g) were purchased from the Institute of laboratory animal science (Beijing, China), feeding with unlimited water and food in the temperature-controlled room. Rat BMSC-Exo was used in *in vivo* experiments. A total of 15 rats were divided into 3 groups and given different treatments until 16 weeks after the surgery: (1) sham operation without removal of kidney tissue (group SHAM, n = 5); (2) 5/6 nephrectomy (group SNx, n = 5); (3) 5/6 nephrectomy plus BMSC-Exo (group SNx + BMSC-Exo, n = 5, tail vein injection, 150 $\mu$g/week). Each step of 5/6 nephrotomy was based on the established modified process after anesthesia by pentobarbital [18]. The rat's body weight was measured at baseline and after 16 weeks of nephrotomy surgery, then the rats were euthanized and samples (serum and kidney) for the subsequent experiment were collected.

### *2.5 Rats serum*

Rats' serum samples were collected for analyzing Serum creatinine (Scr) and blood urea nitrogen (BUN) levels via an automatic biochemical analyzer (AU5421, Olympus, Tokyo, Japan).

### *2.6 HE and Masson trichrome staining*

Kidney tissues were fixed in 10% formalin, then processed for paraffin embedding and at 4 $\mu$m thicknesses. After the procedure of de-paraffinizing, under the guidance of Hematoxylin and Eosin staining (HE) kit and Masson's Trichrome staining kit (Solarbio, Beijing, China), the kidney specimens were stained. The histopathological and fibrosis observation were picked by optical microscope (Olympus, Tokyo, Japan).

### *2.7 Immunohistochemistry*

The sectioned and embedded specimens of kidney tissue were gradually going through dewax, dehydrate, anti-

gen repair and block peroxidase activity. Then the specimens were blocked with 2% bovine serum albumin. Subsequently, primary antibodies against fibronectin (1:100, ab2413, Abcam, Cambridge, UK), Collagen-I (1:200, ab34710, Abcam, Cambridge, UK) and $\alpha$-SMA (1:200, ab5694, Abcam, Cambridge, UK) were added to incubate overnight. Secondary antibody specific for primary antibody was added on the second day for 1 h. At last, the specific proteins were colored by 3,3′-diaminobenzidine tetrahydrochloride (DAB) kit (P0203, Beyotime, Shanghai, China) and the nucleus was colored by hematoxylin. The quantification of immunohistochemistry was performed by Image J software (National Institutes of Health, NIH, Bethesda, MD, USA).

### *2.8 Western blot*

Total protein was extracted from cells or tissues using the Radio Immunoprecipitation Assay (RIPA) lysis buffer and kept at 4 °C for 30 min with vortex shock every 10 min. The primary antibodies against Collagen-I (1:1000, ab34710, Abcam, Cambridge, UK), $\alpha$-SMA (1:1000, ab5694, Abcam, Cambridge, UK), E-cadherin (1:1000, ab40772, Abcam, Cambridge, UK), Smad 7 (1:500, ab216428, Abcam, Cambridge, UK), Smurf 2 (1:1000, 12024S, Cell Signaling Technology, Boston, USA), GAPDH (1:4000, ab181602, Abcam, Cambridge, UK) were incubated overnight for the first day and specific horseradish peroxidase (HRP)-conjugated secondary antibody were incubated 1 hour for the second day. GAPDH was used to normalize the densitometry values of the targeted protein. The primary antibodies were listed in **Supplementary Table 1**.

### *2.9 Statistical analysis*

Data are shown as mean $\pm$ standard deviation (SD). Statistical analysis was performed using Prism 8.0 software (Graph Pad, San Diego, CA, USA). The significant difference between the two groups was determined by Student's *t*-test and one-way factorial ANOVA. *p*-value less than 0.05 was considered as statistically significant.

## 3. Results

### *3.1 BMSC-Exo were internalized by HRPTEpiC*

In our study, both alizarin red S staining and oil red O staining revealed positive results, indicating the highly differentiated ability of BMSCs (**Supplementary Fig. 1**). Three methods were applied to identify exosomes secreted by BMSCs. The transmission electron microscope (TEM) indicated that the extracellular vesicles of the samples were "bowl-shaped" (Fig. 1A). NTA showed that the particle size of BMSC-Exo was approximately 30–150 nm (Fig. 1B). Western blot was used for detecting the exosome marker (CD9, CD63, and CD81) and the nuclear marker (Histone H3), the results showed that the expression of CD9, CD63, and CD81 were enriched in the isolated fractions while Histone H3 were absent (Fig. 1C). Subsequently, we aimed to demonstrate the internalization of exosomes. PKH67 labeled BMSC-Exo were added to HRPTEpiC and incubated for different times (30 min, 24 h), and the labeled exosomes (green fluorescence) were found around the nucleus after being incubated for 24 h (Fig. 1D).

### *3.2 BMSC-Exo improved renal function and histomorphological damage in the 5/6 SNx rat*

To evaluate the effect of BMSC-Exo on renal function and histomorphological damage, a stable 5/6 nephrectomy rat model was established. The kidney of the group SHAM had a normal broad bean-like morphology with a smooth surface. The size of the kidney was significantly bigger under the administration of BMSC-Exo compared with SNx group after 16 weeks of the surgery, indicating BMSC-Exo treatment improved the atrophy of the kidney (Fig. 2A). Moreover, HE staining showed that the regular structure of the kidney was destroyed and displayed glomerular sclerosis, tubular vacuoles, interstitial fibrosis and inflammatory cell infiltration (Fig. 2B) in the SNx group. With the continuous treatment of BMSC-Exo, the aforementioned histological changes were significantly improved, leading to the recovery of renal tissue after injury (Fig. 2B). To further validate the protective role of exosomes, Scr (Serum Creatine), BUN (Blood urea nitrogen) and body weight were measured. Versus the SHAM group, Scr and BUN levels were raised in the SNx group which significantly decreased after administration of BMSC-Exo. For the SHAM group, SNx group and the SNx + Exo group, the level of Scr was 53.01 $\pm$ 3.256, 110.9 $\pm$ 5.465, 72.53 $\pm$ 3.361 respectively, paralleled with the same trend of BUN (16.65 $\pm$ 4.571, 37.55 $\pm$ 1.005 and 19.60 $\pm$ 0.6809). Moreover, the body weight of SNx + Exo group was higher than that of the SNx group (Fig. 2C). The decreased Scr and BUN levels and the increased body weight caused by BMSC-Exo treatment suggesting the explicit therapeutic effect on histomorphological damage and renal function.

### *3.3 BMSC-Exo alleviates renal fibrosis in the 5/6 SNx rats*

To evaluate the effect of BMSC-Exo on renal fibrosis, the Masson-trichrome staining, immunohistochemical staining and western blot were performed. As shown in Fig. 3A, the blue area indicated collagen generation (fibrotic area) was significantly increased after 5/6 nephrectomy. However, the application of BMSC-Exo through tail vein injection significantly reduced the blue-stained collagen deposition area. Consistent with the pathological changes, immunohistochemical staining showed that the positive staining of fibronectin, Collagen-I and $\alpha$-SMA were significantly decreased in the SNx + Exo group compared with the SNx group (Fig. 3B). Moreover, the protein expression level of fibrotic markers Collagen-I and $\alpha$-SMA were decreased while E-cadherin expression was increased after the application of BMSC-Exo compared with

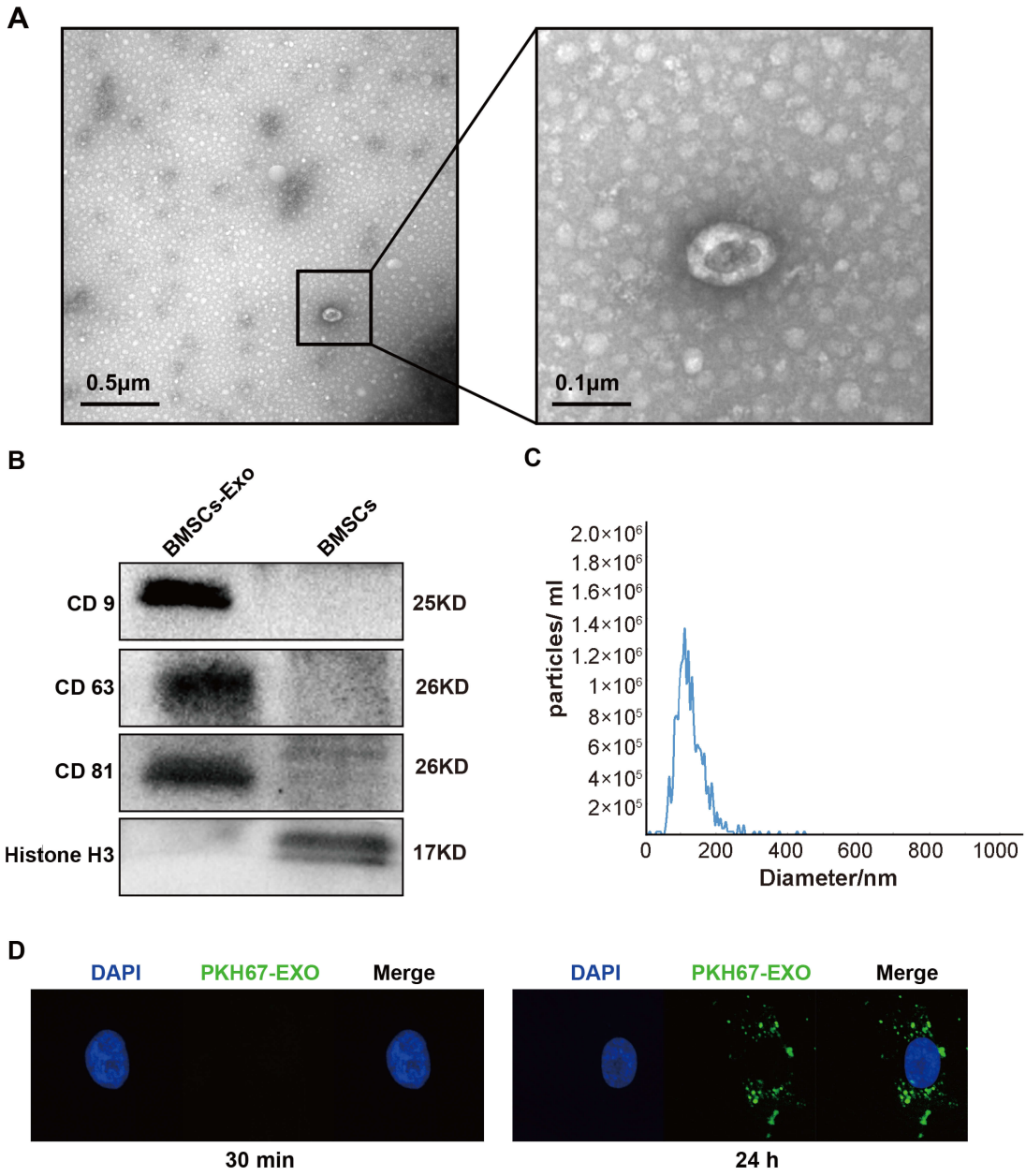


**Fig. 1. Identification and internalization of BMSC-Exo.** (A) TEM revealed the ultrastructure of BMSC-Exo (left panel scale bar = 0.5 $\mu$m, right panel scale bar = 0.1 $\mu$m). (B) BMSC-Exo was analyzed by NTA. (C) Exosome markers (CD9, CD63, and CD81) and the nuclear marker (Histone H3) were detected by Western Blot. (D) The internalization of labeled-BMSC-Exo (green fluorescence) by HRPTEpiC.

SNx group (Fig. 3C). With the evidence above, the therapeutic effect of BMSC-Exo on renal fibrosis is reliable.

### *3.4 BMSC-Exo alleviates TGF-β1-induced fibrosis in HRPTEpiCs*

TGF-$\beta$1 plays a crucial role in the progression of fibrosis. To further demonstrate the anti-fibrotic effect of BMSC-Exo, HRPTEpiCs were incubated with 10 ng/mL TGF-$\beta$1 (TGF-$\beta$ group) or with TGF-$\beta$1 plus 100 ug/mL BMSC-Exo (TGF-$\beta$ + Exo group) for 48 hours. The ovoid HRPTEpiC was elongated and turned into a spindle shape after TGF-$\beta$1 incubation observed under the microscope. The results of western blot showed that TGF-$\beta$1 stimulation evidently increased the expression of $\alpha$-SMA, Collagen-I and decreased the expression of E-cadherin; which were reversed by the administration of BMSC-Exo (Fig. 4A). The results emphasized that BMSC-Exo treatment alleviated TGF-$\beta$1-induced fibrosis *in vitro*.

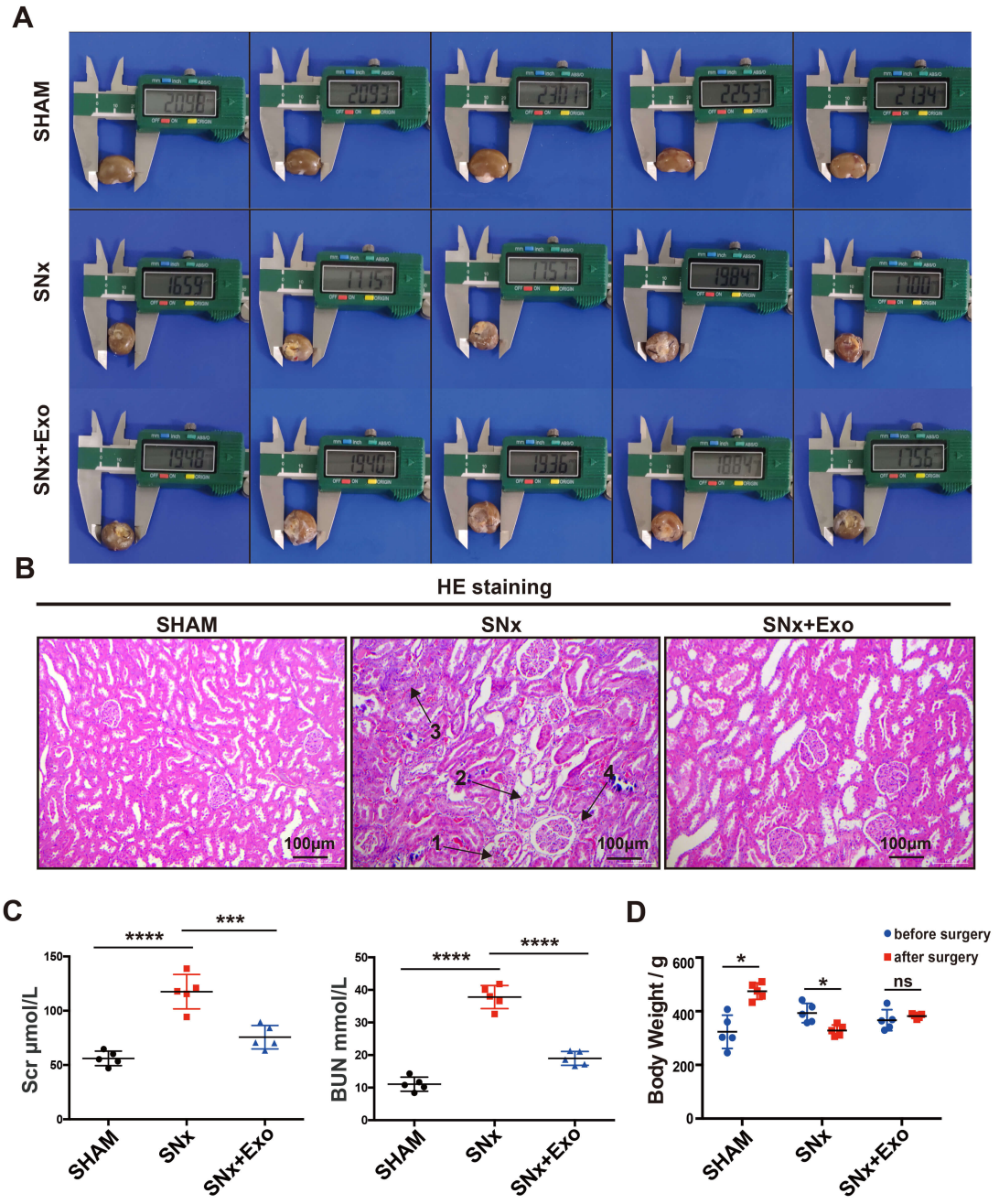


**Fig. 2. BMSC-Exo administration improved renal function and histomorphological damage in the SNx rat.** (A) Images of kidney in different treatment groups. (B) The structure of kidneys was observed under HE staining (scale bar = 100 $\mu$m. Black Arrow 1: glomerular sclerosis; Black Arrow 2: tubular vacuoles; Black Arrow 3: interstitial fibrosis; Black Arrow 4: inflammatory cell infiltration. (C) Comparison of Scr and BUN concentration in rats. (D) Changes of bodyweight in SHAM group, SNx group and SNx + Exo group. ***$p < 0.001$, ****$p < 0.0001$.

### *3.5 BMSC-Exo improves renal fibrosis via inhibiting TGF-β/Smurf 2/Smad 7 pathway*

As we have mentioned in the Introduction section, Smad 7 and Smurf 2 play an important role in TGF-$\beta$ signaling pathway. In addition, the role of Smurf 2 under different pathophysiology context remains complicated and need to be further clarified. In this study, the two molecules Smad 7 and Smurf 2 were measured by western blot both *in vivo* and *in vitro*. Firstly, we demonstrated that the expression of Smad 7 was obviously decreased while Smurf 2 was increased after the establishment of 5/6 nephrectomy compared with the SHAM group. However, the application of BMSC-Exo showed a reverse effect as Smad 7 level was up-regulated and Smurf 2 level was down-regulated compared with the SNx group (Fig. 4B). The same trend was also found in the *in vitro* study. As shown in Fig. 4C, the expres-

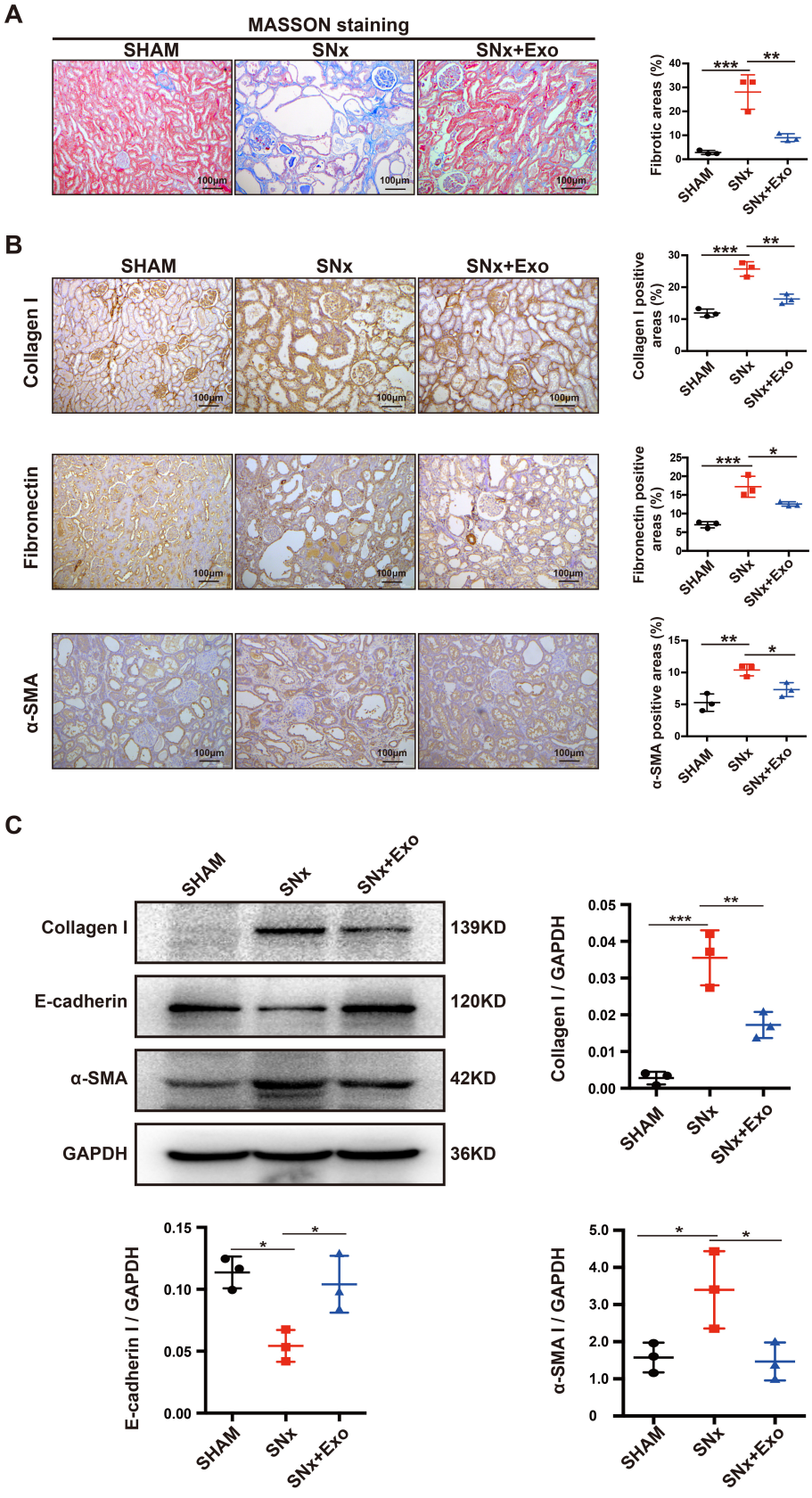


**Fig. 3. BMSC-Exo alleviated renal fibrosis in the SNx rats.** (A) MASSON staining of rat renal tissues (scale bar = 100 $\mu$m). (B) The expression of Collagen-I, Fibronection and $\alpha$-SMA was detected by immunohistochemical staining (scale bar = 100 $\mu$m). (C) Protein expression level of Collagen-I, $\alpha$-SMA and E- cadherin in rats was detected by western blot. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

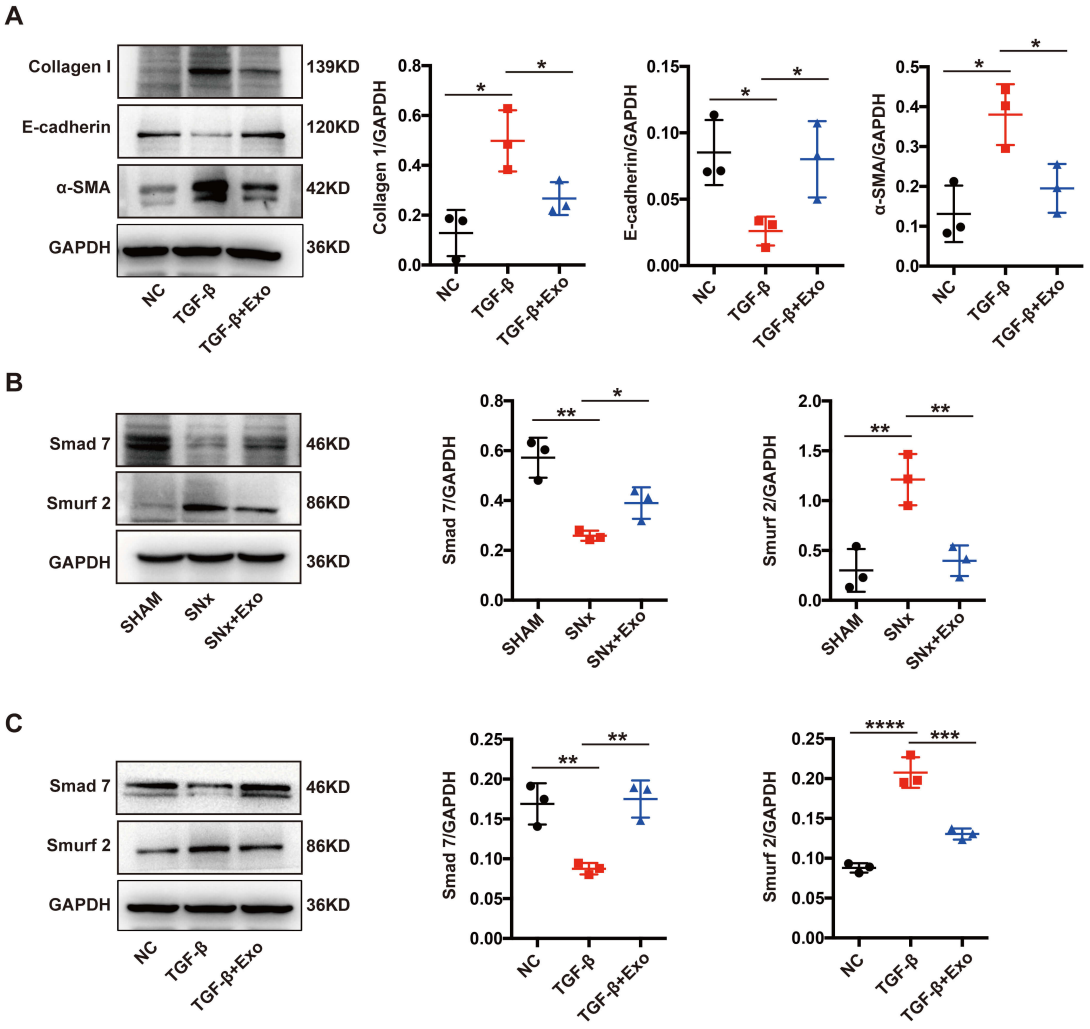


**Fig. 4. BMSC-Exo alleviated renal fibrosis through Smurf 2/Smad 7 axis.** (A) Protein expression level of Collagen-I, $\alpha$-SMA and E- cadherin in HRPTEpiC was detected by western blot. (B) The expression of Smad 7 and Smurf 2 was detected in rats under treatment of BMSC-Exo. (C) The expression of Smad 7 and Smurf 2 was detected in HRPTEpiC after administration of BMSC-Exo. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

sion of Smad7 was significantly decreased while Smurf 2 was increased after the incubation of TGF-$\beta$1, which could be reversed by BMSC-Exo. These results confirmed that BMSC-Exo could partly improve renal fibrosis through regulating Smurf 2/Smad 7 axis.

We further verify the characteristic of BMSC-Exo on Smurf 2/Smad 7 axis. Firstly, the siRNA that specifically targeted Smurf 2 (si-Smurf 2) was used to knock down the expression of Smurf 2. As shown in Fig. 5A, the protein expression of Smurf 2 was significantly decreased by si-Smurf 2, while the expression of Smad 7 was increased. Furthermore, the administration of BMSC-Exo enhanced these effects.

Next, we detected the expression levels of fibrotic markers Collagen-I, $\alpha$-SMA and E-cadherin after silencing Smurf 2. The results demonstrated that after transfection of si-Smurf 2, the expression of Collagen-I, $\alpha$-SMA significantly decreased while E-cadherin expression increased (Fig. 5B), indicating the protective effect of si-Smurf 2 on renal fibrosis. Furthermore, the decreased expression of Collagen-I, $\alpha$-SMA and the increased expression of E-cadherin in group TGF-$\beta$ + si-Smurf 2 were significantly enhanced after administration of BMSC-Exo. Taken together, these aforementioned results confirmed that the BMSC-Exo could inhibit TGF-$\beta$1-induced renal fibrosis, partially, by regulating the Smurf 2/Smad 7 axis (Fig. 6).

## 4. Discussion

Nowadays, the treatment for ESRD is costly and has substantial morbidity. Thus, the new strategy in treating renal fibrosis which is the main pathological process of CKD should be taken into attention. Under this context, the appearance and application of BMSCs-Exo showed a potential role in treating renal fibrosis. Studies have shown that

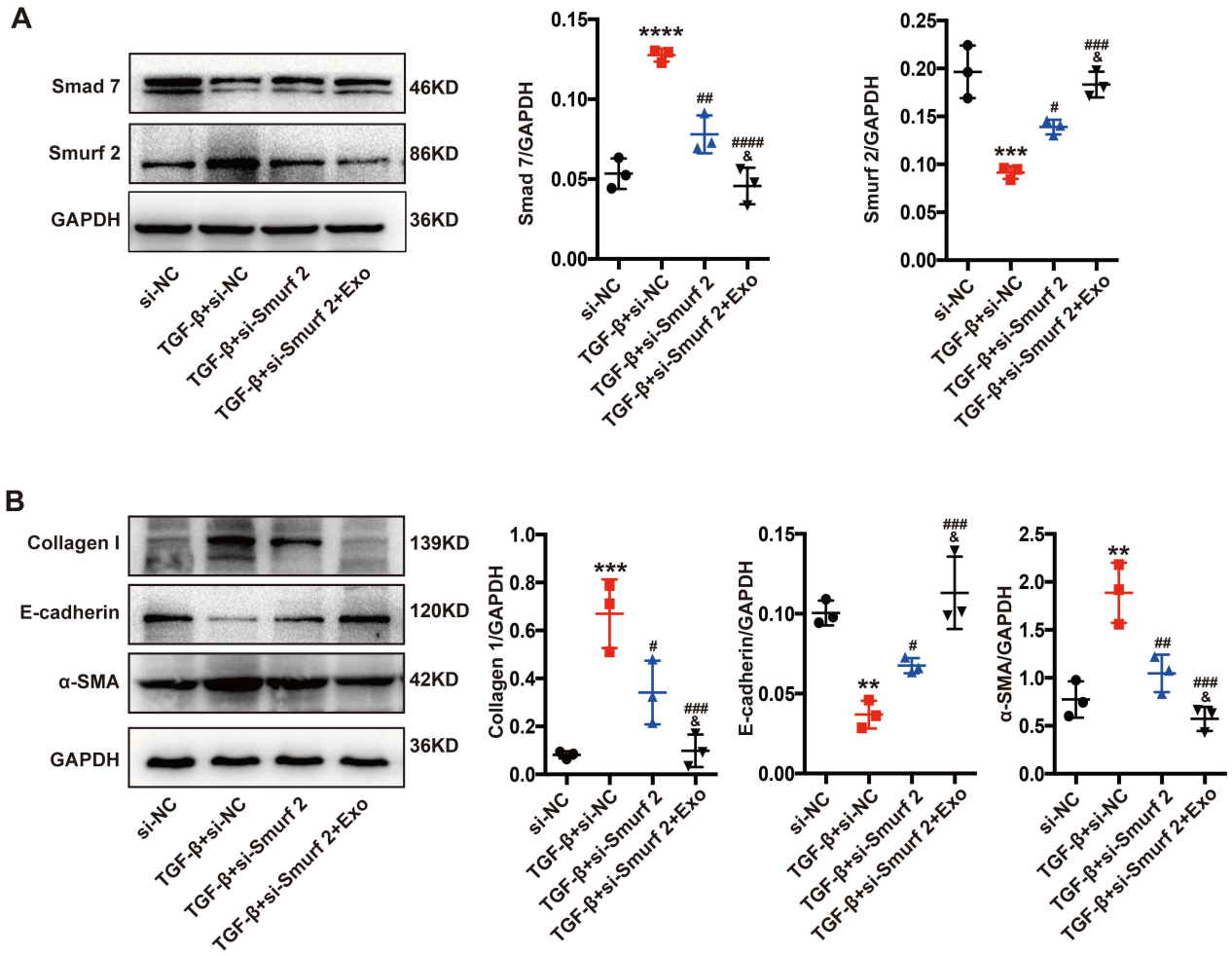


**Fig. 5. BMSC-Exo enhanced the inhibitory effect of si-Smurf 2 on renal fibrosis.** (A) The protein expression level of Smad 7 and Smurf 2 after transfected with si-Smurf 2. (B) The protein expression level of fibrotic markers Collagen-I, $\alpha$-SMA and E-cadherin was measured after transfected with si-Smurf 2. $^{**}p < 0.01$ compared with si-NC group, $^{***}p < 0.001$ compared with si-NC group, $^{****}p < 0.0001$ compared with si-NC group; $\#p < 0.05$ compared with TGF-$\beta$ + si-NC group, $\#\#p < 0.01$ compared with TGF-$\beta$ + si-NC group, $\#\#\#p < 0.001$ compared with TGF-$\beta$ + si-NC group, $\#\#\#\#p < 0.0001$ compared with TGF-$\beta$ + si-NC group; $\&p < 0.01$ compared TGF-$\beta$ + si-Smurf 2.

MSC-exosomes from different sources have completely different functions [19]. However, the research on the effect of BMSC-Exo on renal fibrosis is still limited.

Exosomes are 30–150 nm microvesicles with the lipid bilayer, which contains abundant quantities of mRNA, LncRNA and microRNA to exert multiple effects [20,21]. Exosomes secreted by MSCs have been proved to have therapeutic effects in several complex diseases including in kidney. In the ischemia-induced acute kidney injury model, administration of MSC-derived exosomes evidently alleviates renal injury, improves renal function by inhibiting oxidative stress through regulating NADPH oxidase [22]. Also, human umbilical cord mesenchymal stem cell-derived exosomes can enhance autophagy via modulation ATG16L in the cisplatin-induced acute kidney injury (AKI) [19]. Furthermore, exosomes derived from MSCs also exert an anti-inflammation effect in diabetic nephropathy [17]. In the UUO model, miR-let7C from MSC-derived exosomes could attenuate the process of EMT in TGF-beta induce renal tubular epithelial cells [20]. Collectively, the above studies partly demonstrated the role of exosomes in improve renal injury and fibrosis.

However, because of the complexity of the containing molecules and the polytropic effect in different pathological circumstances, further studies should be done to clarify the therapeutic effect and the underlying mechanisms of BMSC-derived exosomes. In this study, we used primary BMSCs that are more stable and functionally closer to the original tissue to obtain exosomes, but the disadvantage is that they come from a very limited number of donors. A 5/6 nephrectomy rat model and TGF-$\beta$1 treated HRPTEpiCs were established to further investigate the role of BMSC-derived exosomes. In our *in vivo* study, we found that the administration of BMSC-Exo significantly improved at-

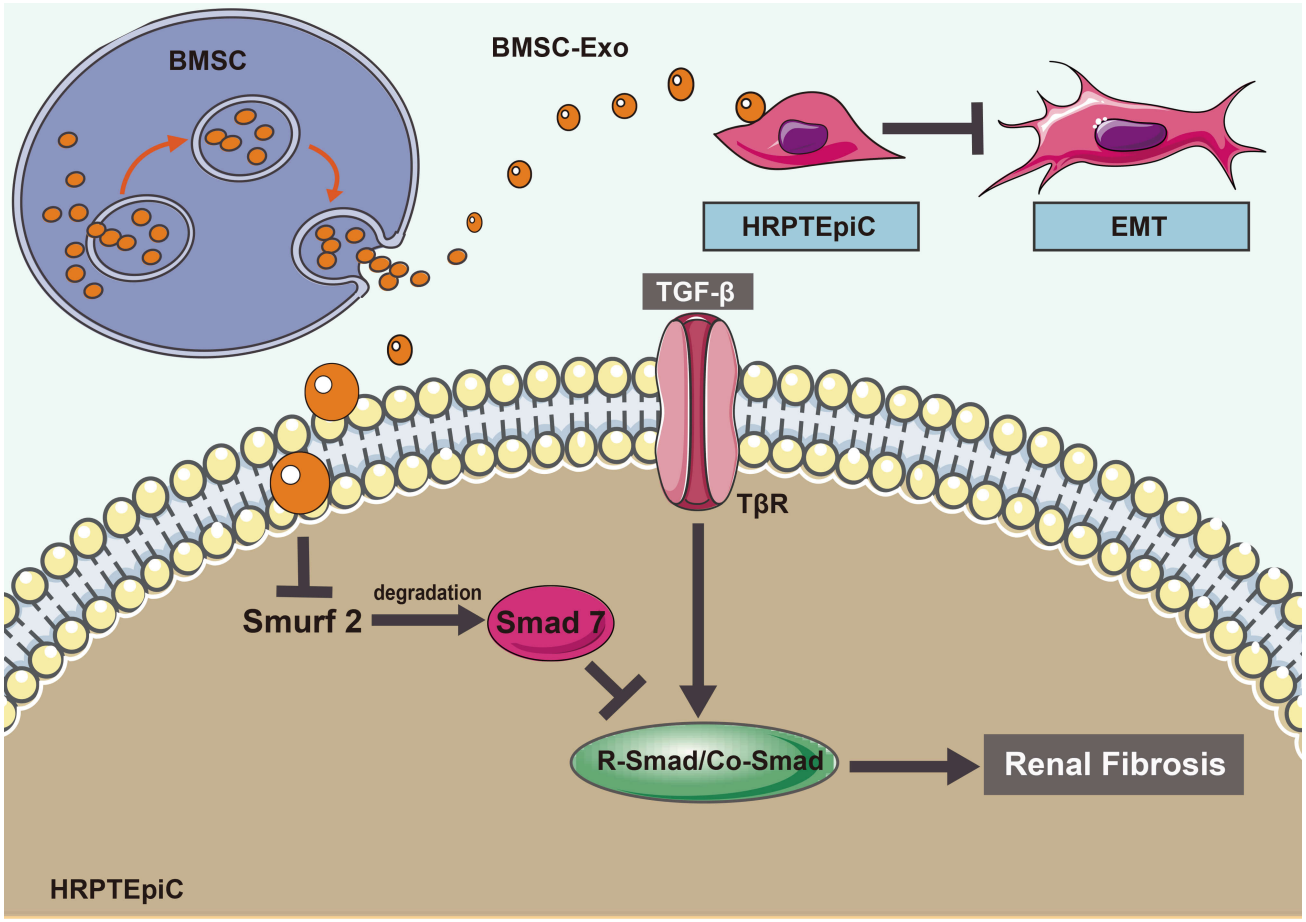


**Fig. 6. Graphical abstract.**

rophic and histomorphological damage of the kidney and alleviated the impaired renal function. Consistent with the previous report, the exosomes also exert an anti-fibrotic role in the 5/6 nephrectomy rat, for reducing the fibrotic area as well as the fibrotic markers. In the nephrectomy rats, TGF-$\beta$1 plays a crucial role in triggering renal fibrogenesis by breaking the balance of ECM synthesis/degradation [23]. TGF-$\beta$1 also promotes renal tubular epithelial cells emerging a fibrotic phenotype thus accelerate the process of renal fibrosis [24]. BMSC-Exo restored the decreased E-cadherin expression and inhibited $\alpha$-SMA and Collagen-I expression compared with TGF-$\beta$1 single treatment. Taken together, BMSC-Exo treatment can inhibit the fibrotic process in the 5/6 nephrectomy rat model and TGF-$\beta$1 treated HRPTEpiCs.

TGF-$\beta$1 acts as a pro-fibrotic molecular mainly through stimulating the downstream Smad signaling pathway to trigger and facilitate subsequent fibrogenesis procession [25]. Previous studies have clearly demonstrated the underlying mechanisms in regulating the TGF-$\beta$1/Smad signaling pathway [3,26]. Among the Smad subfamily, Smad 7 was considered as a negative regulator of TGF-$\beta$1/Smad signaling transduction via blocking the recruitment and phosphorylation of the Smad 2-Smad 3 complex [27]. The crucial role of Smad 7 in renal fibrosis has been proved in different kidney disease models, such as the UUO model, 5/6 nephrectomy model, and ischemia-perfusion model, both of them showed the downregulation of Smad 7 in the injured kidney, on the contrary, restored Smad 7 significantly inhibit renal fibrosis in the above models [28,29]. In our present study, the decreased expression of Smad 7 in the 5/6 nephrectomy rats and TGF-$\beta$1 treated HRPTEpics were observed. However, the administration of BMSC-Exo partly restored the Smad 7 expression and accompanied with decreasing expression of fibrotic markers $\alpha$-SMA and Collagen-I and increasing expression of E-cadherin. As previous research demonstrated, Smad 7 can also exert an anti-inflammation role via upregulating the expression of I$\kappa$B$\alpha$ thus inhibiting the activity of the NF-$\kappa$B signaling pathway [30]. Therefore, the role of BMSC-Exo in inflammation should also be further elucidated in renal fibrosis.

The ubiquitin-proteasome system strictly controlled the downstream action of the Smad subfamily and the signal transduction of TGF-$\beta$ pathway [31]. The subsequent activation of protein degradation by E1-E3 ubiquitin ligases depends on the activation of the ubiquitin-proteasome system. Smad ubiquitination regulatory factor-2 (Smurf 2) is

an E3 ubiquitin ligase that has been proved to physically interact with Smad 7 to induce its ubiquitination and degradation [32,33]. Furthermore, Smurf 2 could also recruit intranuclear Smad 7 and form Smurf 2-Smad 7 complex then binding to TGF-$\beta$ type I receptor and leading the subsequent protein degradation, finally inhibit the TGF-$\beta$/Smad signaling pathway [34]. Thus, under different pathological contexts, the role of Smurf 2 in regulating TGF-$\beta$/Smad pathway is still controversial. In our study, the results showed that the expression of Smurf 2 was significantly inhibited and Smad 7 was increased by BMSC-Exo both in 5/6 nephrectomy rats and TGF-$\beta$1 induced HRPTEpics. Furthermore, knockdown of Smurf 2 induced upregulation of Smad 7 level and downregulated the expression level of $\alpha$-SMA and Collagen-I, and these effects could be enhanced by BMSC-Exo. With the evidence above, we indicated that BMSC-Exo has an inhibitive effect on renal fibrosis, to some extent, by regulating the Smurf 2/Smad 7 axis. Although the benefits of BMSC-Exo in treating fibrotic diseases have been proved in several studies as well as ours, some problems are remained to be further solved, such as the long-term safety of the exosomes and the optimal timing for the treatment of CKD.

## 5. Conclusions

In summary, BMSC-Exo could improve renal fibrosis in 5/6 nephrectomy rats and inhibit TGF-$\beta$1-induced fibrotic changes of HRPTEpics, to some extent, via antagonism of the Smurf 2/Smad 7 axis. This study not only reveals the significant role of the Smurf 2/Smad 7 axis in regulating renal fibrosis but also expands our understanding of the regulatory effects of BMSC-Exo on diseases with complex mechanisms such as CKD-VC.

## Abbreviations

BMSC-Exo, Bone marrow mesenchymal stem cells-derived exosome; SNx, 5/6 subtotal nephrotomy; HRPTEpiCs, human renal proximal tubular epithelial cells; CKD, Chronic kidney disease; TGF-$\beta$1, Transforming growth factor-$\beta$1; R-Smad, receptor Smad; Co-Smad, common mediator Smad; I-Smad, inhibitory Smad; Smurf 2, Smad ubiquitination regulatory factor 2; MSCs, Mesenchymal stem cells; TEM, Transmission electron microscope; si-Smurf 2, siRNA targeting Smurf 2; Scr, Serum creatinine; BUN, Blood urea nitrogen; HE, Hematoxylin and Eosin staining; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; AKI, acute kidney injury; ESRD, End-stage renal disease; UUO, unilateral ureteral obstruction.

## Author contributions

WL, XC and YL provided the concept and designed the study; YL and YG performed experiments; XC and YL interpreted the results; YL prepared figures; XC drafted the manuscript; XC, YL, WG and WL edited and revised the manuscript. All authors approved the final version of the manuscript.

## Ethics approval and consent to participate

All procedures involving animal samples were approved and supervised by the animal ethics committee of Beijing Friendship Hospital, Capital Medical University (21-1005). The treatment of animals in all experiments conforms to the ethical standards of experimental animals.


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## Conflict of interest

The authors declare no conflict of interest.

## Supplementary material

Supplementary material associated with this article can be found, in the online version, at https://www.imrpress.com/journal/FBL/27/1/10.31083/j.fbl2701017.

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