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Nuclear receptor 4A1 ameliorates UUO-induced renal fibrosis by inhibiting the PI3K/AKT pathway

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As an ultra-early response gene, Nuclear receptor 4A1 (NR4A1) has been reported to be involved in the development of various diseases through various pathological pathways, but its specific mechanism in chronic kidney disease (CKD) is unknown currently. Our study showed that the expression of NR4A1 was reduced in unilateral ureteral obstruction (UUO) mice and it could exacerbate UUO-induced renal pathological injury when knocked down NR4A1 in UUO mice. We found that the knockdown of NR4A1 could promote angiogenesis, renal inflammation, and cell apoptosis to aggravate renal fibrosis induced by UUO. As an agonist of NR4A1, Cytosporone B (Csn-B) could inhibit the renal fibrosis by attenuating angiogenesis, renal inflammation and cell apoptosis. In addition, the PI3K/AKT pathway was activated with NR4A1 knockdown in vivo and in vitro experiments. In conclusion, our study demonstrates that NR4A1 can ameliorate renal fibrosis. Furthermore, we speculate that its underlying mechanism may be related to the activation of PI3K/AKT pathway according to our present results.

Keywords NR4A1, Chronic kidney disease, Renal fibrosis, PI3K/Akt

Chronic kidney disease (CKD) is characterized by kidney damage or the glomerular filtration rate is lower than 60 mL/min for at least 3 months, which is a global health problem[1]. With the aging population and changes in disease spectrum, the incidence of CKD has been increasing over the years[2]. It has become a serious global public health problem with high mortality and disability rates[3].

Renal fibrosis is a common pathological pathway from CKD to chronic renal failure, including renal interstitial fibrosis and glomerulosclerosis[4]. It is essential for the treatment of CKD to inhibit the progression of renal fibrosis[5]. Previous studies have shown that the progress of renal fibrosis is closely related to inflammation, oxidative stress and angiogenesis[6–9]. However, its specific mechanism is still unknown. Therefore, it is significantly for clinical treatment of CKD to clarify the underlying mechanism of renal fibrosis.

Nuclear receptor 4A (NR4A) subfamily is a long family of nuclear receptor superfamily, including three members: NR4A1, NR4A2 and NR4A3[10–13]. Among of these, NR4A1 is a member of the nuclear hormone receptor superfamily. As an immediate early gene, it is involved in a variety of pathophysiological processes including the occurrence and development of CKD[14–16]. Accumulated evidence suggests that NR4A1 is a CKD susceptibility gene. Its deficiency can exacerbate renal tubular atrophy, tubular cast and interstitial fibrosis accompanied by extensive infiltration of immune cells in mice[17]. Previous studies have shown that transforming growth factor-β1(TGF-β1) is the main driver of renal fibrosis[18,19], while NR4A1 is an endogenous inhibitor of TGF-β1[12]. During the process of fibrosis, TGF-β1 is upregulated to inhibit the transcriptional activity of NR4A1 by translocating it from the nucleus to the cytoplasm, while the upregulation of NR4A1 can inhibit fibrosis by repressing the expression of TGF-β1[12,20]. It indicates that there is a negative feedback loop between NR4A1 and TGF-β1. Our previous study has proved that NR4A1 was down-regulated in renal tissue of UUO rats with the transcriptome assay[21], and it could improve renal fibrosis by up-regulating the expression of NR4A1[22].

Here, we mainly tested the effect of NR4A1 on renal fibrosis. We used adenovirus to knock down the expression of NR4A1 in mice, thereby exploring its effect on angiogenesis, cell apoptosis, and inflammation, which were related to the progression of renal fibrosis. This study will provide a basis for basic research and clinical drug development in the future.

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Materials and methods

Animal experiments

C57BL/6 J mice (6–8 weeks) were purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. C57BL/6 J mice were randomly divided into 7 groups.

Ethical approval

All experiments were carried out in accordance with recommendations for the Care and Use of Laboratory Animals in the National Institutes of Health Guidelines. Animal care followed the criteria of the Ethics Committee of Hebei University of Chinese Medicine. The authors complied with the ARRIVE guidelines.

Animal models and treatment

To perform unilateral ureteral obstruction (UUO) surgery, an incision was made in the left mid abdomen to free the left ureter after the animals were anesthetized with isoflurane inhalation. Ligation was performed at the upper and middle one-third of the ureter, and a renal fibrosis model was replicated by cutting in the middle. The sham group only dissociated the ureter but did not ligate or cut it[23]. The animals were placed in the animal room after waking up.

To knock down NR4A1 in animals, we used the adenovirus injection in mice[24]. The adenovirus vectors were purchased from Hanbio Biotechnology Co., Ltd (Cat#: AD21062310, AP21070906). The adenovirus was delivered into the kidneys of experimental mice by intrarenal injection. Mice were anesthetized and fixed to expose the left kidney. Purified lentivirus was extracted with micro-injector. The control group was injected with the same amount of sh-con. The needle was inserted from the lower pole of the kidney along the longitudinal axis and carefully pushed to the upper pole at a depth of approximately 0.5 cm, avoiding renal veins, arteries and the ureters. Then the needle was slowly withdrawn and injected at the same time. The kidney turned white after the injection, which indicated that the injection was successful. We then waited 2–3 s before removing the needle. The sh-NR4A1 were injected into the kidneys of mice for 1 week after UUO surgery for 1 week in the sh-con + UUO group and the sh-NR4A1 + UUO group. However, the sh-con were injected into the kidneys of mice for 1 week after sham surgery for 1 week in the sh-con + sham group and the sh-NR4A1 + sham group. Then the mice were sacrificed 14 days after the operation, and the renal tissues were collected for the subsequent histological and biochemical analysis.The mice were intraperitoneal injected with Cytosporone B (Csn-B) dissolved in dimethyl sulfoxide (DMSO) once a day for 2 weeks in the Csn-B + UUO group, with a dosage of 10 mg/kg/day.

Histomorphology and immunohistochemical staining

Paraffin kidney sections were prepared by a routine procedure. Histopathological observation of kidney: after being fixed with 4% paraformaldehyde solution, the kidney tissue was routinely dehydrated, paraffin embedded, and made into continuous sections with a thickness of about 4 μm. Perform HE, Sirius red, and Masson staining. HE staining (inflammatory cell infiltration and tubulointerstitial changes) were semi-quantitatively graded by two investigators. The two items were scored as 0 (minor), 1 (minor), 2 (moderate), and 3 (severe), with a total score ranging from 0 to 6. A semiquantitative analysis of Masson and Sirius red staining was performed according to the percentage of the collagen positive areas. Image analyses were performed using Image J 6.0 software.

Immunohistochemically staining (IHC) was performed with kidney sections according to the established protocol. After routine dewaxing and dehydration of paraffin sections, repair antigens were carried out, and after natural cooling to room temperature, endogenous peroxidase was eliminated with 3% H_2O_2. After blocking the goat serum, the primary antibody VEGFA (Abcam, 1:200, Cat#: ab52917), CD105 (Abcam, 1:200, Cat#: ab230925),TNF-α (Abclonal, 1:100, Cat#:A0277), IL-1β (Abcam, 1:200, Cat#: ab283818) was incubated at 4 °C overnight, and the second antibody IgG was added dropwise at room temperature (1:300). After PBS cleaning, DAB staining was performed, followed by hematoxylin re staining, acidification and dehydration before sealing, and observed under the microscope.

Immunofluorescence staining (IF)

Tissues were embedded in OCT, sectioned, washed in PBS, antigen repair, cooled at room temperature, washed in PBS, goat serum blocked for 30 min, plus monoantibody α-SMA (1:200) for 4 °C overnight. The next day after PBS washing, Lotus Tetragonolobus LectinI (LTL, used to label the renal tubules, 1:100, Lot #: 2856098) was incubated for 30 min, After PBS washing, DAPI staining was added for 10 min and sealing.

Tunel staining

Following the conventional steps of dewaxing and dehydration of paraffin slices, PBS was washed 3*5 min. Protease K (Solarbio,Cat#: P1120) was used to break the membrane (37 ℃, 1 h), and then the tunel buffer was prepared according to the ratio of the One-step Tunel Apoptosis Assay Kit (Abbkine, Cat#: KTA2010). It was sealed in dark at 37 ℃ for 1 h, and PBS was washed 3 times for DAPI staining. After PBS cleaning, the film was sealed and observed under a fluorescence microscope.

Cell culture and treatment

Human proximal renal tubular epithelial cells (HK-2) were cultured in Dulbecco's Modified Eagle's medium (DMEM, Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, CA, USA), 1% penicillin and 1% streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. HK-2 cells were grown in culture flasks at 80% to 90% confluence and harvested with trypsin/EDTA. Then the cells were seeded into 60 mm petri

dishes (Corning) at a certain ratio. After 24 h of culture, these cells were treated with or without TGF-β1(10 ng/mL) for another 24 h.

Lentiviral transfection

The cells were seeded into a 6 cm cell culture vessel to adjust the cell density, which corresponded to 50–60%. Then the cells were infected with the supernatant of the media containing the lentivirus. Next, the lentivirus containing the shRNA against NR4A1 (sh-NR4A1), or the negative (blank) control (shNC) was transfected into the cells using a 10^8 TU/mL titer. The cells were then cultured in a 5% CO_2 cell incubator and harvested after transfection for 48 h. Finally, the transfection efficiency was verified and characterized. Extract protein perform relevant testing.

Western blot

Total protein was extracted by RIPA lysis, protein concentration was determined by BCA method, equal mass sample loading method was used, 10% SDS-PAGE gel electrophoresis was used for separation, and wet transfer to PVDF membrane. Shake the bed at room temperature for 1 h and incubate, the primary antibody α-SMA (1:1000), Vimentin (Abcam,1:500,Cat#: ab92547), Collagen I (Proteintech,1:300,Cat #:14695-1-AP), VEGFA (1:500), CD105 (1:500), TNF-α (1:100), Bcl-2 (Proteintech,1:500,Cat #:26593-1-AP), Bax (Proteintech,1:500,Cat #:50599-2-Ig), NF-κB(Proteintech,1:500,Cat#:10745-1-AP), GAPDH (Proteintech, 1:3000, Cat#:10494-1-AP), PI3K(Proteintech, 1:500, Cat#:60255-1-1g), PI3K (phospho Tyr467/199, Immunoway, 1:500, Cat#:YP0225), Akt (Proteintech, 1:500, Cat#:60203-2-Ig), phospho-Akt (Ser473, Proteintech, 1:500, Cat#:60203-2-Ig) at 4 °C overnight. After washing the film, the second antibody (Proteintech, 1:3000, Cat#:SA00001-2, Cat#:SA00001-1) was incubated for 1 h, and the ECL luminescent solution developed and exposed for imaging.

Statistical analysis

The quantitative data shown in this study are representative of at least three experiments and are expressed as means ± standard deviation (means ± SD). IBM SPSS 23.0 software (https://www.ibm.com/products/spss-statistics) (IBM, Armonk, NY, USA) was used for statistical processing, $P < 0.05$ as the criterion for statistically significant differences.

Results

Knocking down NR4A1 can aggravate renal pathological damage in UUO mice

To validate the expression of NR4A1 in renal fibrosis, we replicate the animal model of renal fibrosis using UUO and first performed pathological staining to observe the mold formation. Masson staining showed that significant collagen deposition was observed in the UUO group, indicating successful moulding ($P < 0.05$) (Fig. 1A). We first used western blot to detect the level of NR4A1 protein. The results showed that NR4A1 was significantly downregulated in UUO mice ($P < 0.05$) (Fig. 1B), which is consistent with our previous results.

To further validate the role of NR4A1 in renal fibrosis, we injected sh-NR4A1 virus into mice kidneys to specifically knock down the expression of NR4A1 in kidney tissue. Firstly, we tested the knockdown effect of NR4A1, and western blot results showed that the expression of NR4A1 was significantly reduced after virus injection($P < 0.05$) (Fig. 2B). We used HE, Masson and Sirius red staining to detect the renal pathological damage of mice. The HE staining results showed that there were renal tubular atrophy and a large number of inflammatory cell infiltration in UUO mice. However, knocking down NR4A1 resulted in more significant tubular atrophy and increased infiltration of inflammatory cells ($P < 0.05$) (Fig. 1C). Masson staining results showed that it would lead to most higher collagen deposition in renal interstitial after knocking down NR4A1($P < 0.05$) (Fig. 1C). The results of Sirius red staining were consistent with these of Masson staining ($P < 0.05$) (Fig. 1C). The above results indicate that NR4A1 expression is reduced in UUO rats and mice, and knockdown of NR4A1 can exacerbate kidney damage.

Knocking down NR4A1 can exacerbate renal fibrosis in UUO mice

We also examined the impact of NR4A1 knockdown on renal fibrosis to validate the role of NR4A1 in CKD. We estimate the extent of renal fibrosis by detecting the expressions of α-SMA, Vimentin, and Collagen I. The results of IF showed that UUO-induced renal fibrosis occurred near the renal tubules, and NR4A1 knockdown could significantly increase UUO-induced α-SMA expression (Fig. 2A). Western blot showed that the expressions of Vimentin and Collagen I were significantly increased in UUO mice. However, knocking down NR4A1 can significantly exacerbate UUO-induced renal fibrosis ($P < 0.05$) (Fig. 2B). Taken together, these results demonstrate that knockdown of NR4A1 can exacerbate renal fibrosis in UUO mice.

Knocking down NR4A1 can induce angiogenesis in UUO mice

IHC results showed that the expressions of VEGFA and CD105 were upregulated in UUO mice, but NR4A1 knockdown could increase their expressions ($P < 0.05$) (Fig. 3A). Additionally, the results of western blot showed that the expressions of VEGFA and CD105 were up-regulated with knockdown of NR4A1 ($P < 0.05$) (Fig. 3B). The above results indicate that NR4A1 can inhibit angiogenesis to alleviate renal fibrosis.

Knocking down NR4A1 can induce renal inflammation in UUO mice

We measured the expressions of TNF-α, IL-1β and NF-κB. The results of IHC and western blot showed that UUO mice exhibit significant inflammatory responses, but their expressions were significantly increased with knocking down NR4A1 ($P < 0.05$) (Fig. 4A and B). These results elucidate that NR4A1 can induce inflammatory response to exacerbate renal fibrosis.

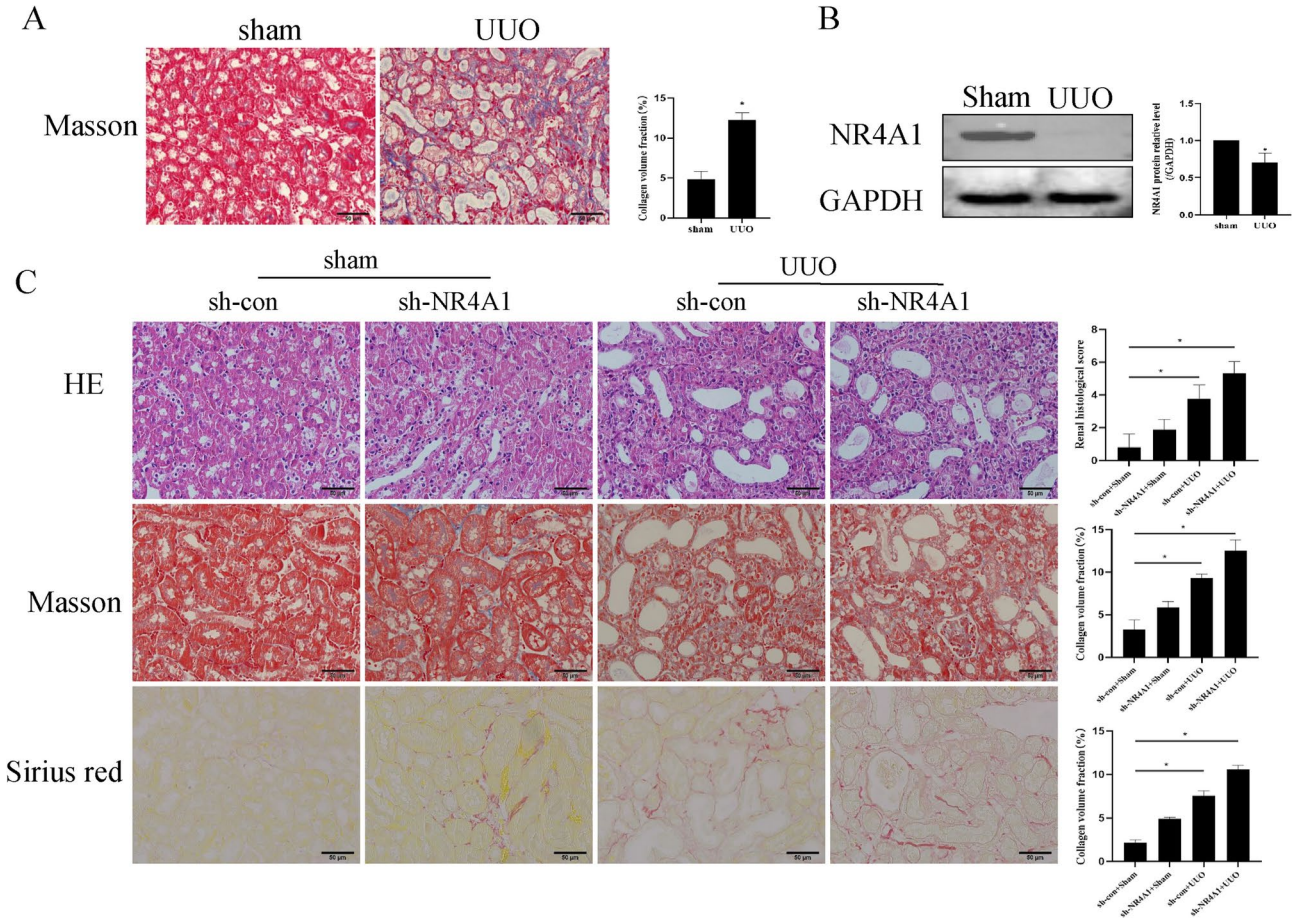

Fig. 1. Knocking down NR4A1 can aggravate renal pathological damage in UUO mice. (**A**) Masson to examine collagen deposition (n = 6). Masson is blue. (**B**) Western blot to examine NR4A1expression in mice (n = 3). (**C**) HE to examine morphological changes and inflammatory cell infiltration (n = 6). Masson was used to examine collagen deposition (n = 6). Sirius red was used to examine collagen deposition (n = 6). Masson is blue, Sirius red is red. The data are presented as the mean ± SD, $^{*}P < 0.05$.

Knocking down NR4A1 can promote cell apoptosis in UUO mice

We used Tunel staining to analyze apoptosis, and applied western blot to detect the expressions of Caspase-3 and Bcl-2. The results of Tunel staining showed that knocking down NR4A1 could significantly increase the number of apoptotic cells ($P < 0.05$) (Fig. 5A). In addition, western blot results showed that it could upregulate the expression of Caspase-3 but downregulate the expression of Bcl-2 with knocking down NR4A1 ($P < 0.05$) (Fig. 5B). Taken together, these results declare that NR4A1 may improve renal fibrosis by inhibiting cell apoptosis.

Csn-B ameliorates UUO-induced renal fibrosis by inhibiting inflammatory response, angiogenesis, and apoptosis

The above studies have shown that knockdown of NR4A1 can aggravate renal fibrosis by promoting angiogenesis, inflammatory response, cell apoptosis, etc. To further verify the role of NR4A1, Csn-B was administrated in UUO mice. The results of HE showed that the renal tubules were arranged and infiltrated with little inflammatory cells in the sham group. Compared with it, the UUO group showed a large number of inflammatory infiltrations, renal tubular atrophy, deformation, and dilation. However, Csn-B could decrease UUO-induced the inflammatory cell infiltration ($P < 0.05$) (Fig. 6A). The results of Masson and Sirius red staining showed that UUO-induced massive collagen deposition in the kidneys, while Csn-B could reduce UUO-induced collagen deposition ($P < 0.05$) (Fig. 6A). Besides, we used IF and western blot to test the expression of fibrosis related genes. The results showed that the α-SMA and Vimentin expressions were significantly increased in the UUO group than that in the sham group. However, Csn-B could significantly inhibit UUO-induced the expressions of α-SMA and Vimentin ($P < 0.05$) (Fig. 6B and C). The above results indicate that Csn-B can reduce the pathological kidney damage and improve renal fibrosis by activating NR4A1.

According to our previous results, we also examined the angiogenesis, inflammation, or apoptosis related proteins expression. The results showed that the CD34, CD105, VEGFA, TNF-α, NF-κB and Bax expression were significantly increased, and Bcl-2 expression was significantly decreased in the UUO group compared with the Sham group. However, Csn-B could significant inhibit UUO-induced the expressions of CD34, CD105, VEGFA,

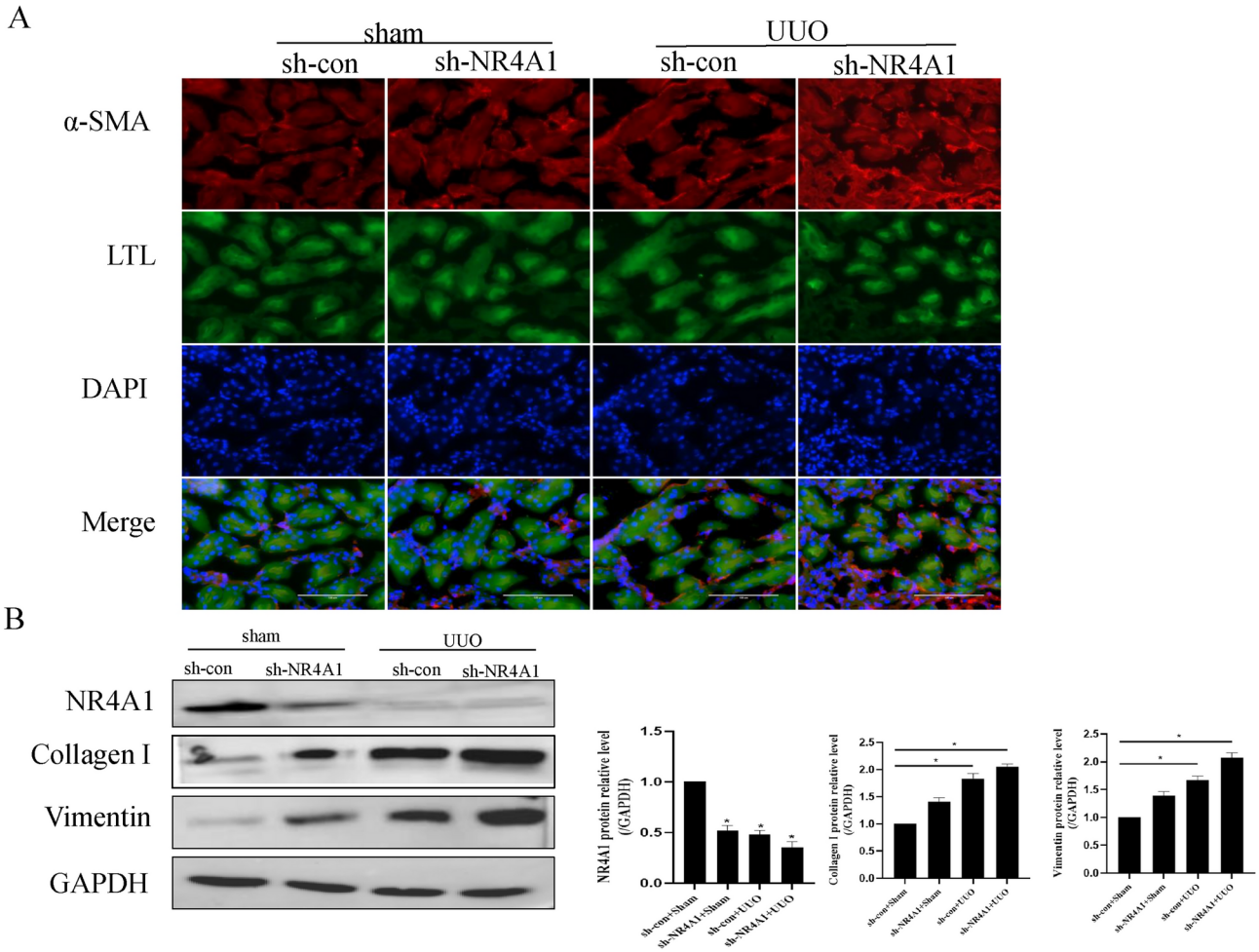

Fig. 2. Knocking down NR4A1 can exacerbate renal fibrosis in UUO mice. (**A**) Immunofluorescence staining to examine α-SMA and LTL expression in NR4A1 knocking down mice (n = 6). Red is α-SMA, Green is LTL. (**B**) Western blot to examine NR4A1, Vimentin and Collagen I expression in knocking down mice (n = 3). The data are presented as the mean ± SD, $^{*}P < 0.05$.

TNF-α, NF-κB, and Bax, and upregulate UUO-induced the Bcl-2 expression ($P < 0.05$) (Fig. 7A and B). The above results declare that Csn-B can ameliorate renal fibrosis by inhibiting inflammatory response, angiogenesis, and apoptosis.

NR4A1 may regulate the PI3K/Akt signaling pathway to improve UUO or TGF- β1-induced renal fibrosis

We first carried out the expressions of PI3K/Akt and p-PI3K/p-Akt in renal fibrosis models. Western blot analysis showed that the levels of p-PI3K/p-Akt were significantly increased in the UUO mice($P < 0.05$) (Fig. 8A). To further validate the above results, we further tested their levels with NR4A1 knockdown or not in mice. The results showed that the levels of p-PI3K and p-Akt were upregulated when NR4A1 was knocked down ($P < 0.05$) (Fig. 8B). These results clarify that NR4A1 may exacerbate renal fibrosis by regulating the PI3K/Akt signaling pathway.

To further verify the role of PI3K/Akt pathway, we performed validation in vitro. The results showed that the levels of p-PI3K and p-Akt were significantly up-regulated in HK-2 cells treated with TGF-β1. Nevertheless, Csn-B could significantly decrease the TGF-β1-induced PI3K/Akt phosphorylation levels in HK-2 cells ($P < 0.05$) (Fig. 8C). In addition, the knockdown of NR4A1 could significantly upregulate the TGF-β1-induced p-PI3K and p-Akt levels ($P < 0.05$) (Fig. 8D).

Discussion

Chronic nephritis, diabetes and hypertension are the common causes for CKD. Its high risk factors include infection, nephrotoxic drugs, immune diseases and old age[25]. In recent years, it has become a public health problem, and its incidence rate is 10.8% in China[1,2]. Although the clinical efficacy of therapeutic methods for CKD has been improved, it also brings huge economic burden to patients and society with long-term therapy. Renal fibrosis is the common final outcome underlying the progression of CKD to end-stage renal disease[5]. It can

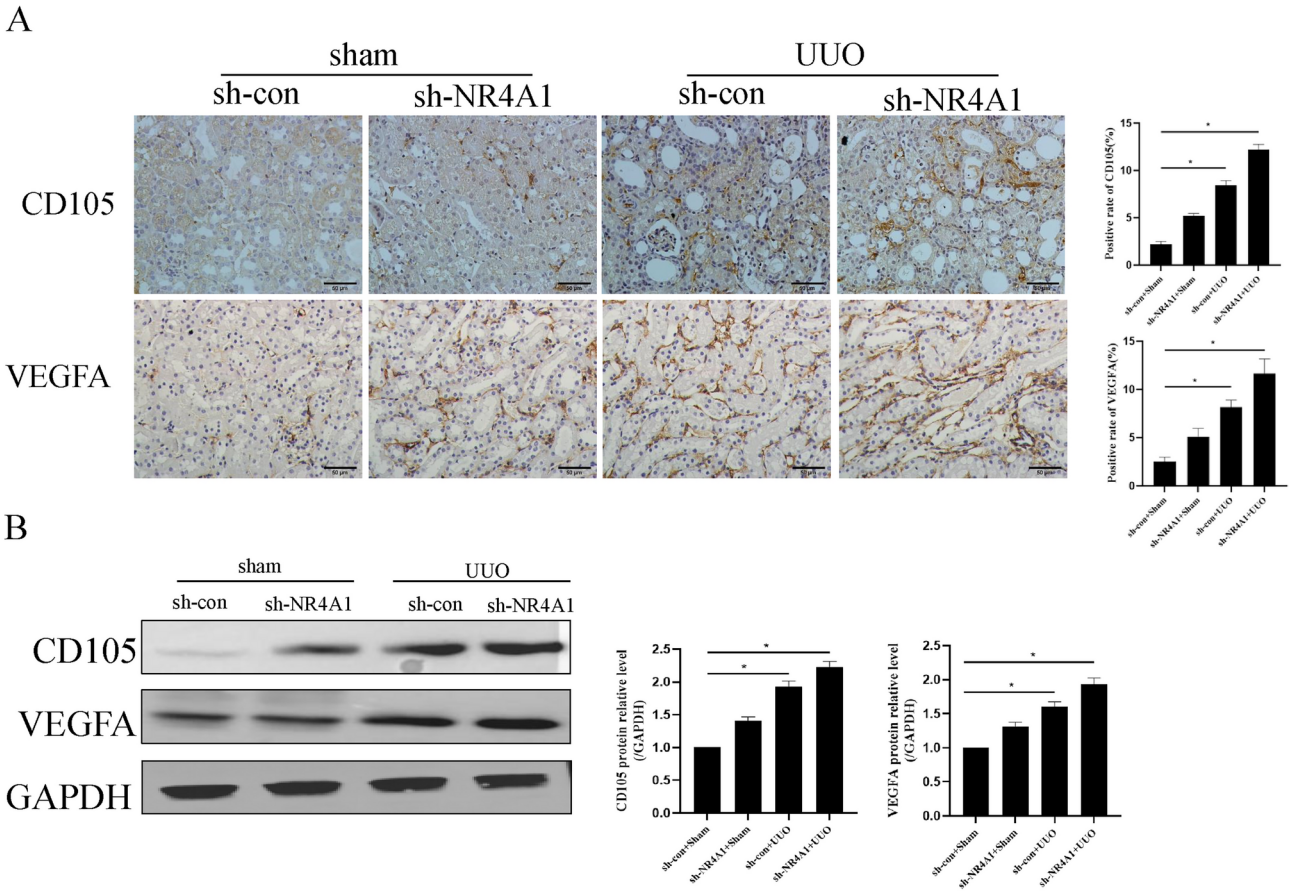

Fig. 3. Knocking down NR4A1 can induce angiogenesis in UUO mice. (**A**) Immunohistochemical staining to examine CD105 and VEGFA expression in knocking down mice(n = 6). (**B**) Western blot to examine CD105 and VEGFA expression in knocking down mice (n = 3). The data are presented as the mean ± SD, $^{*}P < 0.05$.

effectively improve CKD by inhibiting or even reversing renal fibrosis. It has been proved that the progression of renal fibrosis can be promoted by many pathological processes, such as inflammation, apoptosis, angiogenesis[7,8]. Therefore, the renal fibrosis can be repressed with the suppression of these pathological processes. However, its mechanism is still unknown.

Nuclear receptor superfamily can regulate various physiological processes and play an important role in many pathological processes[26]. NR4A1 is a key member of the nuclear receptor superfamily, which plays an important role in maintaining cell homeostasis[10]. As a super early response gene, it is involved in various physiological and pathological processes, such as cell differentiation, apoptosis, proliferation, inflammation, and metabolism[7,14–16]. NR4A1 can act as a susceptibility gene of CKD to play an important role in the occurrence and development of CKD[17,27]. In addition, as a endogenous inhibitor of TGF-β1, NR4A1 can play an important role in renal fibrosis[12]. It would aggravate the kidney injury to accelerate the progress of CKD with the decrease of NR4A1. Moreover, knocking down NR4A1 can exacerbate renal function damage and TGF-β1 induced renal fibrosis[20].

Our previous study showed that NR4A1 was significantly downregulated in a UUO rat model[21]. Upregulation of NR4A1 can improve the mitochondrial function of UUO rat by increasing the activity of mitochondrial complexes (I-V) and key metabolic enzymes, thereby improving renal fibrosis[22]. In this study, our results also showed that NR4A1 expression was reduced, which is consistent with our previous findings. In order to further validate the role of NR4A1 in renal fibrosis, we injected purified adenovirus into the kidneys of mice to knock down NR4A1 in the kidneys or administrated Csn-B in UUO mice to activate NR4A1. Our results show that knocking down NR4A1 can induce renal pathological damage and exacerbate renal fibrosis. But Csn-B can inhibit renal pathological damage and ameliorate renal fibrosis.

The persistent chronic inflammatory response is the main cause of the occurrence and development of CKD[28,29]. As a classic inflammation related signaling pathway, TNF-α is significantly upregulated in CKD, while it can effectively improve renal fibrosis by inhibiting its expression[30]. NF-κB is a classical inflammatory factor. IL-1β, as an important pro-inflammatory factor in innate immunity, also plays an important role in CKD[31]. Our results show that TNF-α , IL-1β and NF-κB expression are significantly elevated after NR4A1 knockdown in UUO mice. However, Csn-B could significant inhibit UUO-induced the expression of TNF-α, IL-1β and NF-κB.

Angiogenesis refers to the process of migration, proliferation and budding of vascular endothelial cell migration to form new blood vessels on the basis of the original blood vessels[32]. Vascular endothelial growth factor (VEGF) is released to induce angiogenesis with hypoxia and inflammation[33]. However, the new blood

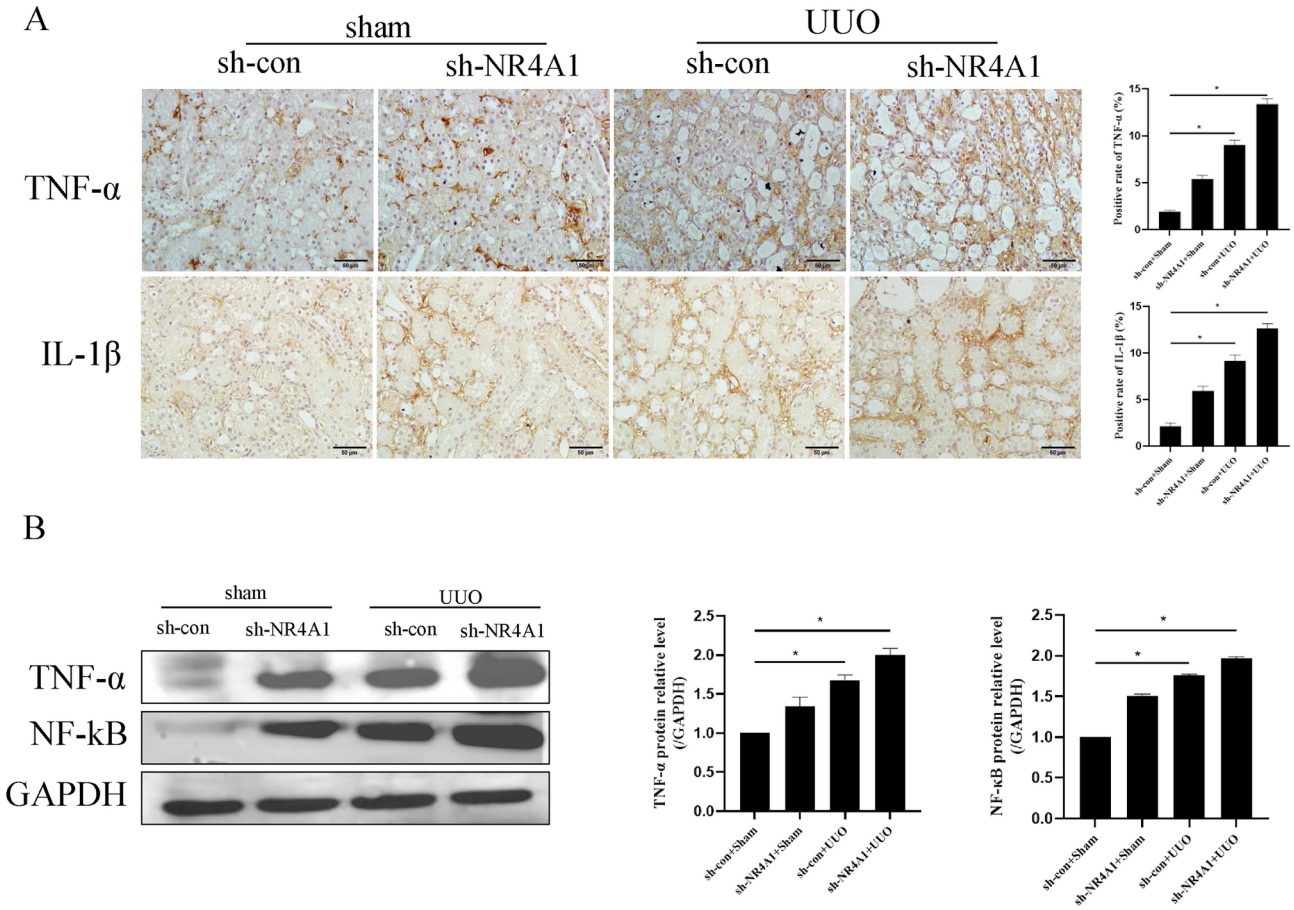

Fig. 4. Knocking down NR4A1 can induce renal inflammation in UUO mice. (**A**) Immunohistochemical to examine TNF-α and IL-1β expression in knocking down mice (n = 6). (**B**) Western blot to examine TNF-α and IL-1β expression in knocking down mice (n = 3). The data are presented as the mean ± SD, $^{*}P < 0.05$.

vessels are immature, disordered and easy to leak, leading to an increase in exudate, which will further aggravate the hypoxia and inflammatory damage of local tissues[5]. Previous studies have shown that angiogenesis can aggravate renal fibrosis[34]. Therefore, we detected the related indicators of angiogenesis, such as VEGFA[35] and CD105 (labeled with neovascularization)[36]. Our results show that VEGFA and CD105 expression are significantly increased after NR4A1 knockdown in UUO mice. But Csn-B could significant inhibit UUO-induced the expression of CD34, CD105 and VEGFA.

Apoptosis can induce renal tubulointerstitial fibrosis by exacerbating renal tubular atrophy and extracellular matrix deposition, thereby accelerating the progression of CKD[37,38]. Therefore, we used Tunel staining to analyze apoptosis, and applied western blot to detect the expressions of Caspase-3 and Bcl-2[39]. Our results show that Caspase-3 expression are significantly elevated, and the number of apoptotic cells increase after NR4A1 knockdown in UUO mice. However, Csn-B could significant inhibit UUO-induced the expression of Bax and upregulate UUO-induced Bcl-2 expression. All of these results indicate that NR4A1 may affect renal fibrosis by regulating the pathological process inflammatory damage, angiogenesis and apoptosis. However, its exact mechanism is not clear, which needs furthermore experiments to explore it.

PI3K/Akt signaling pathway is an important and quite complex signaling pathway that plays a role in many cellular processes crucial to the human body, including cell cycle, cell survival, inflammation, metabolism, and apoptosis[40–43]. Research has shown that this signaling pathway plays a crucial regulatory role in various physiological functions of CKD[43–45]. Our RNA-sequencing results showed that NR4A1 may regulate PI3K/Akt to exacerbate renal fibrosis[21]. To further validate these results, we detected the expression of genes related to this signaling pathway. Our results have shown that the levels of p-PI3K and p-Akt are significantly increased in UUO rats and mice as well as HK-2 cells treated with TGF-β1. It could enhance their increased levels with the NR4A1 knockdown, but Csn-B could inhibit TGF-β1-induced their levels in HK-2 cells. All of these results indicated that there was a significant negative correlation between the levels of p-PI3K/p-Akt and that of NR4A1. According to our results, we found that NR4A1 may participate in the occurrence and development of renal fibrosis by downregulating the PI3K/Akt signaling pathway, which in turn inhibits angiogenesis, inflammatory response, and cell apoptosis. In summary, our research demonstrates that NR4A1 can inhibit renal fibrosis by suppressing inflammatory response, cell apoptosis, and angiogenesis. Here, we preliminary speculate that it

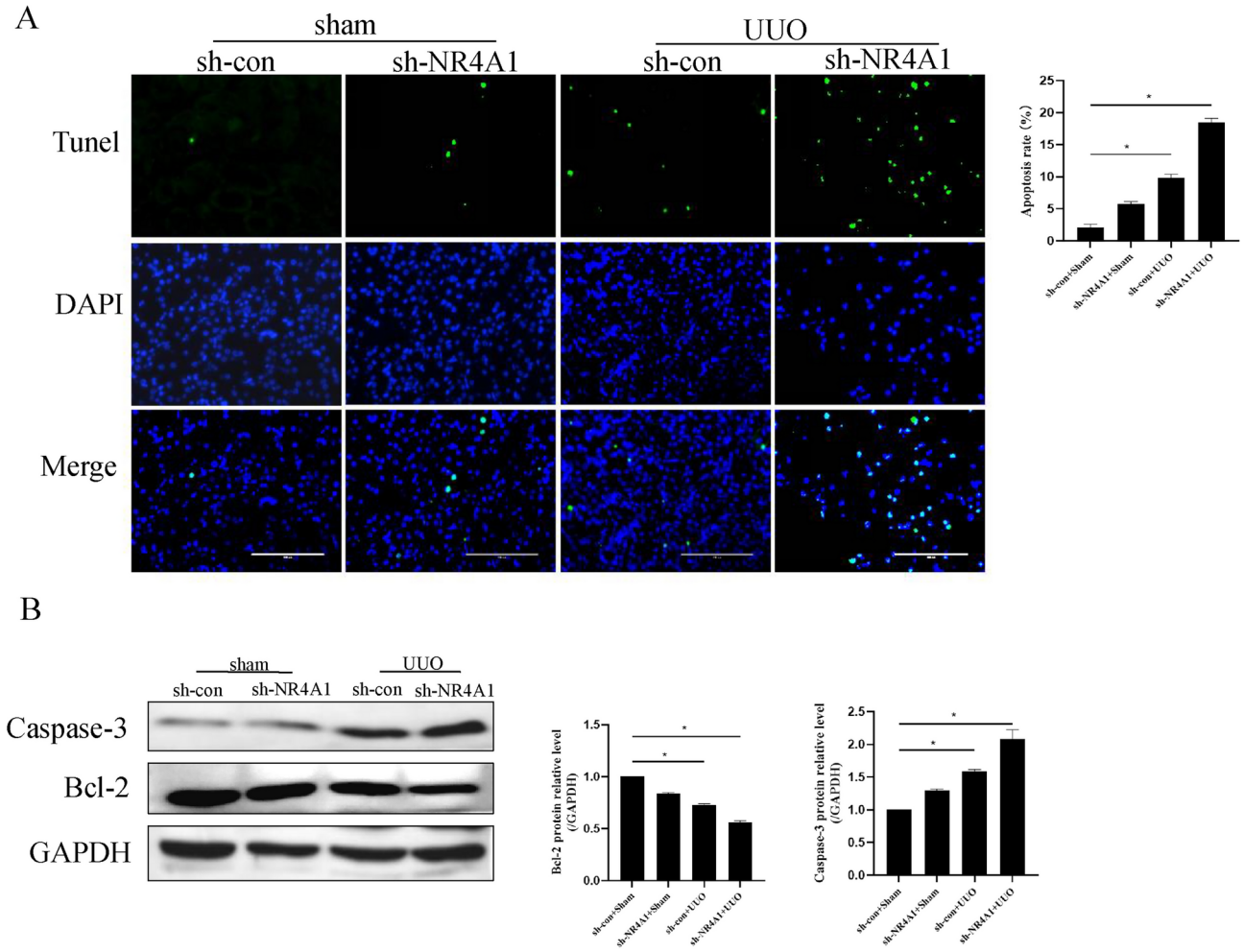

Fig. 5. Knocking down NR4A1 can promote cell apoptosis in UUO mice. (**A**) Tunel staining to examine apoptosis (n = 6). (**B**) Western blot to examine Caspase-3 and Bcl-2 expression in knocking down mice (n = 3). The data are presented as the mean ± SD, $^{*}P < 0.05$.

may regulate PI3K/Akt signaling pathway to play its role in renal fibrosis. Although it needs to conduct more experiments for supporting this, this study provides a new and novel mechanism for the treatment of CKD.

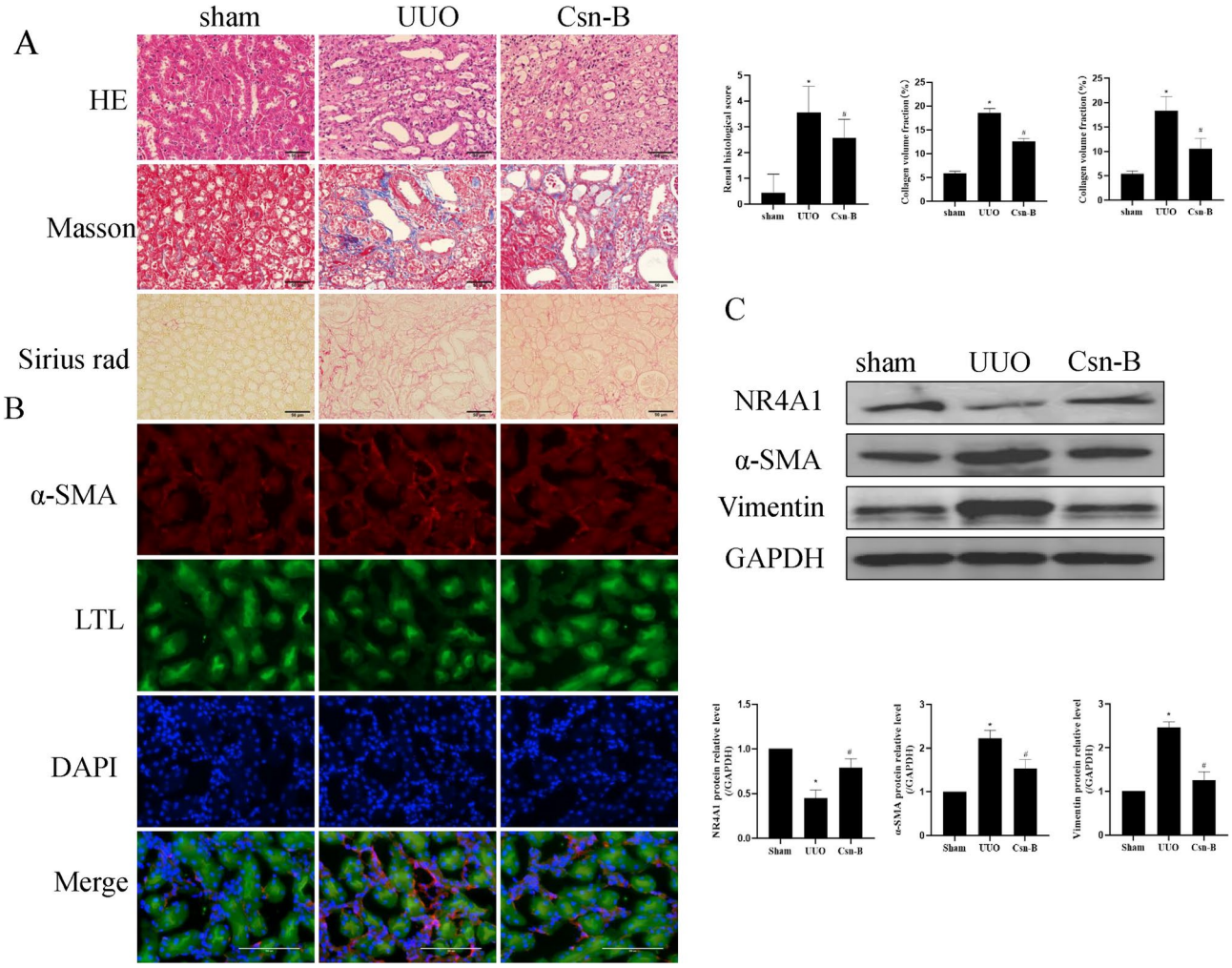

Fig. 6. Csn-B ameliorates renal pathological injury and reduces renal fibrosis in UUO mice. (**A**) HE to examine morphological changes and inflammatory cell infiltration (n = 6).Masson was used to examine collagen deposition (n = 6).Sirius red was used to examine collagen deposition (n = 6). (**B**) Immunofluorescence staining to examine α-SMA and LTL expression in mice (n = 6). Red is α-SMA, Green is LTL. (**C**) Western blot to examine α-SMA,Vimentin and NR4A1 expression in mice (n = 3). The data are presented as the mean ± SD, $^{*}P < 0.05$ vs. the sham group. $^{\#}P < 0.05$ vs. the UUO group.

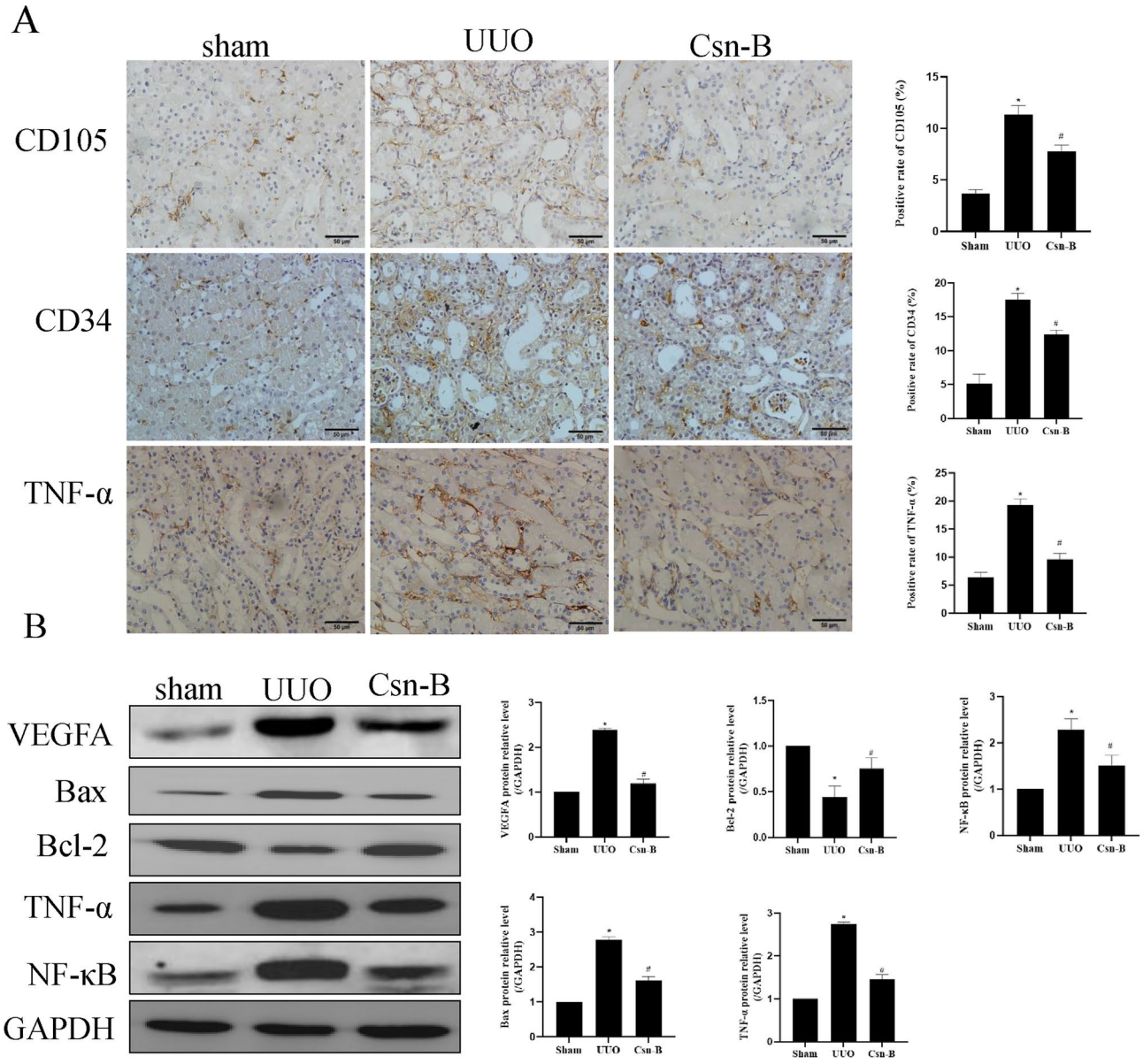

Fig. 7. Csn-B ameliorates renal pathological injury and reduces renal fibrosis in UUO mice. (**A**) Immunohistochemical to examine CD105,CD34 and TNF-α expression in mice (n = 6). (**B**) Western blot to examine VEGFA,Bax,Bcl-2,TNF-α and NF-κB and expression in mice (n = 3). The data are presented as the mean ± SD, $^{*}P < 0.05$ vs. the sham group. $^{\#}P < 0.05$ vs. the UUO group.

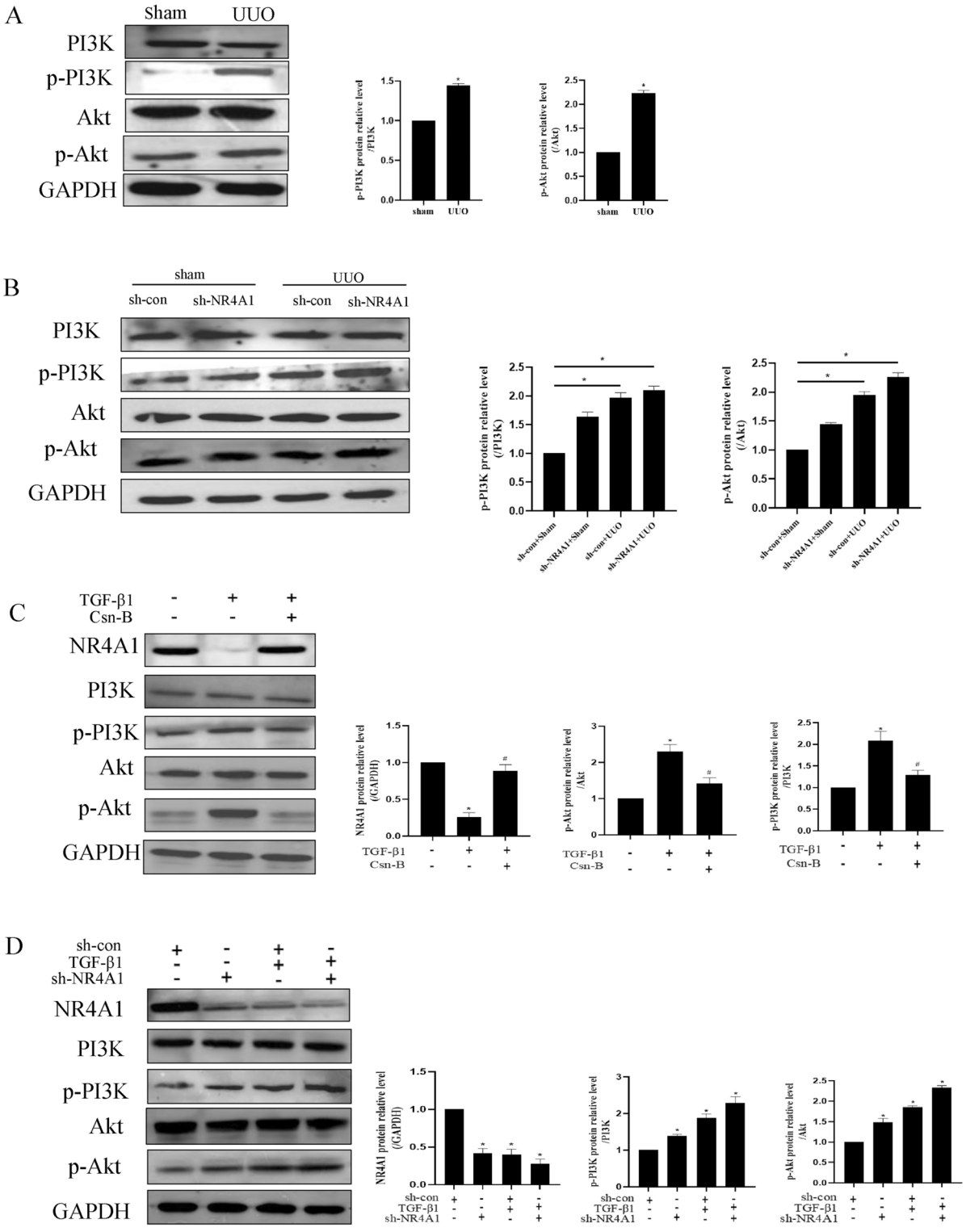

Fig. 8. NR4A1 may regulate the PI3K/Akt signaling pathway to improve renal fibrosis in UUO mice. (**A**) Western blot to examine PI3K/Akt expression in mice (n = 3). (**B**) Western blot to examine PI3K/Akt expression in NR4A1 knocking down mice (n = 3). (**C**) Western blot to examine PI3K/Akt expression in HK-2 (n = 3). (**D**) Western blot to examine PI3K/Akt expression in HK-2 (n = 3). The data are presented as the mean ± SD, $^{*}P < 0.05$ vs. the sham/sh-con/Con group. $^{\#}P < 0.05$ vs. the sh-NR4A1/TGF-β1 group.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable.

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Author contributions

HS W, Y Z and XT W designed the study. HS W, ZH W and C X performed animal experiments. HS W, ZH W, C X and F F performed cell experiments. HS W, Z W and Y Z analyzed data. HS W wrote the manuscript. Y Z and XT W revise the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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