



Nrf2 signaling attenuates epithelial-to-mesenchymal transition and renal interstitial fibrosis via PI3K/Akt signaling pathways

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ABSTRACT

Background: Nrf2 constitutes a therapeutic reference point for renal fibrosis and chronic kidney diseases. Nrf2-related signaling pathways are recognized to temper endothelial-to-mesenchymal transition (EMT) in fibrotic tissue. Nevertheless, the mechanism by which Nrf2 mitigates renal interstitial fibrosis is imprecise.

Methods: The relationship between Nrf2 and renal interstitial fibrosis was investigated using the unilateral ureteral obstruction (UUO) model of Nrf2^{-/-} mice. The mice were separated into four groups, based on the treatment and intervention: Nrf2^{-/-} + UUO, Nrf2^{-/-} + Sham, WT + UUO and WT + Sham. Histological examination of renal tissue following the hematoxylin-eosin and Masson staining was carried out, as well as immunohistochemical staining. Additionally, to confirm the *in vivo* discoveries, *in vitro* experiments with HK-2 cells were also performed.

Results: The Nrf2^{-/-} + UUO group showed more severe renal interstitial fibrosis compared to the WT + UUO, Nrf2^{-/-} + Sham and WT + Sham groups. Furthermore, the manifestations of α -SMA and Fibronectin significantly increased, and the manifestation of E-cadherin considerably decreased in kidney tissues from the group of Nrf2^{-/-} + UUO, compared to the WT + UUO group. The Nrf2 protein level significantly decreased in HK-2 cells, in reaction to the TGF- β 1 concentration. In addition, the overexpression of Nrf2 presented contradictory results. What is more, the PI3K/Akt signaling pathway was discovered to be activated in the proteins extracted from cultured cells, and treated with Nrf2 siRNA and kidney tissues from the Nrf2^{-/-} + UUO group.

Conclusions: The results we obtained demonstrate that Nrf2 signaling pathway may perhaps offset the development of EMT, prompted by TGF- β 1 and renal interstitial fibrosis. Likewise, the anti-fibrotic effect of Nrf2 was imparted by the inactivation of PI3K/Akt signaling. From our discoveries, we deliver new insight related to the prevention and treatment of kidney fibrosis.

1. Introduction

Kidney interstitial fibrosis is closely related to the progressive loss of kidney function, and consequent end-stage kidney disease (Risdon et al., 1968), which is induced by the extreme deposition of extracellular matrix (ECM) in the kidney interstitium, as well as activated fibroblasts and myofibroblasts (Wang et al., 2016; Sakai et al., 2017). Following the deposition of ECM, renal perivascular fibroblasts, interstitial fibroblasts, tubular epithelial cells and endothelial cells transition to mesenchymal cells occurs (Okada et al., 2000; Zeisberg et al., 2008; Yu et al., 1995). As a result of the epithelial-to-mesenchymal transition (EMT) of tubular epithelial cells, at the region of injury, up to a third of

myofibroblasts are formed (Iwano et al., 2002).

The participation and integration of several molecules and various signaling pathways are required at different stages during the EMT, which is a complex and dynamic process. Embryonic development, tumor progression and organ fibrosis (Hay, 1995; Marcucci et al., 2016) necessitate the EMT. EMT is defined as the substantial loss of epithelial cell markers, like E-cadherin, and the procurement of mesenchymal markers, like α -smooth muscle actin (α -SMA), along with the disruption of the tubular basement membrane, and fibroblast incursion accompanied by pro-fibrotic molecules production, like fibronectin and collagen-I (Iwano et al., 2002; Liu, 2004; Kalluri and Neilson, 2003; Zeisberg et al., 2001). In the latest years, consorting EMT with the

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pathogenesis of kidney fibrosis has increasingly caught in attention.

As a member of the “cap ‘n’ collar” basic leucine zipper family, the nuclear factor erythroid2-related factor2 (Nrf2), is a vital orchestrator of cell reactions to oxidative stress, protecting cells against oxidant-induced injury (Ma, 2013; Kay et al., 2011). It has been discovered that Nrf2 binds to antioxidant response elements (AREs). The promoter region of genes encoding antioxidant and phase II detoxifying enzymes, are the location of AREs, which regulate antioxidant, inherent immune and cytoprotective reactions (Ma et al., 2006; Itoh et al., 1999). Nrf2 has attracted great interest as a therapeutic target for kidney fibrosis and chronic kidney diseases. The Nrf2-heme oxygenase-1 (HO-1) system was originally acknowledged as a protective factor in a mouse model of cyclosporine A-induced kidney fibrosis (Shin et al., 2010). Moreover, a purified compound, antroquinonol, with inhibitory effects on nitric oxide creation and inflammatory reactions, was recounted to considerably offset interstitial fibrosis and renal dysfunction, by raising Nrf2 activity in a mouse model of focal segmental glomerulosclerosis (Tsai et al., 2011). Concerning the action mechanism of Nrf2, studies have shown that Nrf2-associated signaling pathways moderate the *in vitro* development of EMT, prompted by the transforming growth factor β 1 (TGF- β 1) (Ryoo et al., 2015, 2014). Nevertheless, the mechanism inherent to the function of Nrf2 in averting renal fibrosis is uncertain, and the current study pursue to answer this question.

Our hypothesis in this study is that, Nrf2 can protect the kidney from interstitial fibrosis, by downregulating EMT progression. The expression of EMT-related proteins was studied in Nrf2-deficient (Nrf2^{-/-}) and wild-type (WT) mice, in order to prove our hypothesis. Additionally, our investigation of the mechanisms underlying the role of Nrf2 in interstitial fibrosis in human renal epithelial cells (HK-2) served as confirmation of the *in vitro* results.

2. Methods and materials

2.1. Ethics statement

The protocol of our study was in line with the Declarations of Helsinki and Istanbul ethical standards, and ratified by the Animal Care Ethics Committee of Nanjing Medical University Affiliated Nanjing Children's Hospital.

2.2. Reagents

Human recombinant TGF- β 1 was procured from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Nrf2, α -SMA, E-cadherin, Snail1, fibronectin and GAPDH were bought from Abcam (Cambridge, MA, USA). Antibodies against phospho-Akt (Ser473) and phospho-PI3K (p85; Tyr458), and the PI3K selective inhibitor wortmannin were procured from Cell Signaling Technology (Beverly, MA, USA). Penicillin-streptomycin and Dulbecco modified Eagle medium (DMEM) were bought from Invitrogen (Life Technologies, Grand Island, NY, USA). Fetal bovine serum (FBS) was bought from Gibco (Carlsbad, CA, USA).

2.3. Cell culture and treatment

Human renal proximal tubule epithelial (HK-2) cells were procured from KeyGEN Biotechnology (Nanjing, Jiangsu, China) and cultured in DMEM complemented with 10% FBS and 1% penicillin-streptomycin at 37 °C in a 5% CO₂ atmosphere. The cells were initially famished in DMEM containing 0.5% FBS during 24 h, in order to study the effect of Nrf2 on the pathogenesis of EMT. Afterwards, the cells were washed twice with PBS at 4 °C, and treated with TGF- β 1 at concentrations of 1, 2 and 5 ng/ml for 48 h (He et al., 2015). To examine the proteins affected, the cells were originally starved in DMEM comprising 0.5% FBS during 24 h. They were washed twice afterwards, with 4 °C PBS and processed with the selective inhibitor wortmannin (0.5 μ M) for 1 h. A

stimulation with TGF- β 1 (5 ng/ml) followed, for 48 h. Total protein and total RNA was extracted from the cells for western blot analysis and quantitative real-time PCR (qRT-PCR). Each experiment defined above was replicated at least three times.

2.4. Western blot assay

The extraction of total proteins from HK-2 cells or kidney tissues was performed, and using a BCA protein assay, the protein concentrations were determined (Thermo Scientific, MA, USA). The protein were divided into equal amounts using 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transmitted to nitrocellulose membranes. Regarding the immunodetection, incubations with primary antibodies against was used to treat the blots, by means of GAPDH (1:200), α -SMA (1:2500), E-cadherin (1:1000), Nrf2 (1:5000), Snail1 (1:1000), TGF- β 1 (1:250), p85 (1:1000), phospho-p85 (1:1000), Akt (1:1000), and phospho-Akt (1:1000). Subsequently, the incubation with an anti-rabbit or anti-mouse secondary antibody (1:1000), was performed. The proportional abundance of proteins was defined according to GAPDH expression, which served as an internal reference, and bands were measured using an Odyssey infrared imaging system (LI-COR Biotechnology, Lincoln, NE, USA).

2.5. Cell transfection

We planned and synthesized siRNA against Nrf2, and its negative control siRNA as well, using GeneChem Biotechnology (Shanghai, China). The Nrf2 primers sequences were as thus: 5'-TCAGCGACGGA AAGAGTATGA-3' (forward) and 5'-CCACTGGTTTCTGACTGGATGT-3' (reverse). Following the manufacturer's guidelines, these siRNAs were transfected into HK-2 cells by Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA). To build the Nrf2 overexpression plasmid pCMV6-Nrf2, the CDS region of the Nrf2 gene was implanted into the pCMV6 plasmid, which was manufactured by GeneChem Biotechnology (Shanghai, China). The negative control used was the pCMV-empty.

2.6. Animal model

Dr. Wei Zhang generously provided the Nrf2^{-/-} mice and their WT littermates (the First Affiliated Hospital with Nanjing Medical University, Nanjing, Jiangsu, China). All the animals were initially procured from the Jackson Laboratory of USA, and preserved in the SPF laboratory of the Animal Core Facility of Nanjing Medical University.

We used a mouse model of unilateral ureteral obstruction (UUO) to test our hypothesis. Interstitial inflammatory cell infiltration, oxidative stress, apoptosis and fibrosis characterizes the UUO model (Soranno et al., 2014; Truong et al., 1996). The mice were harvested on the 7th day, 14th day and 21th day after the surgery, so as to obtain the classical varying histological lesions in the obstructed kidneys (Lopez-Guisa et al., 2011). Regarding UUO's induction, all Nrf2^{-/-} mice and their WT littermates weighing around 20 g were sedated with evertin (125 mg/kg), and the left ureter was ligated through flank incision. Four groups were used to arbitrarily divide all the 60 mice (30 Nrf2^{-/-} mice and 30 WT mice): WT + sham mice (n = 15 for 7, 14 and 21 days), WT + UUO mice (n = 15 for 7, 14 and 21 days), Nrf2^{-/-} + sham mice (n = 15 for 7, 14 and 21 days), Nrf2^{-/-} + UUO mice (n = 15 for 7, 14 and 21 days). The mice in each group were sacrificed on the 7th, 14th or 21th day after UUO induction. The kidneys were harvested. For histological evaluation, half of the harvested kidneys were fixed in 4% buffered formalin, and implanted in paraffin, while for protein evaluation, the other half were kept under -80 °C liquid nitrogen.

2.7. Histological examination and immunohistochemical staining

For the histopathological exams, the kidney tissue sections were

stained with hematoxylin-eosin (HE), and for collagen detection and evaluation, it's the Masson's trichrome staining that was used. In 15 random fields per slide, the level of tubulointerstitial fibrosis was quantitatively determined with fibrosis score, which was autonomously accomplished by two authors (Jun Wang and Haobo Zhu). The proportion of damaged tubules, including tubulitis, atrophy and necrosis, in the total renal tubules, were used to calculate the fibrosis scores of each slide. The highest damage was in 4 score (> 75%) and normal in 0 score (Landolt et al., 2017; Zou et al., 2017).

To determine the distribution and expression of Nrf2, α -SMA and E-cadherin in the harvested kidney tissues, IHC staining assays were performed. Xylene was used to deparaffinized the tissue sections of 5 μ m thickness, and they were rehydrated in a graded series of alcohol. During 30 min, non-specific epitopes were blocked with 5% normal goat serum, and subsequently incubated all-night with primary antibodies against Nrf2 (1:100), α -SMA (1:200) and E-Cadherin (1:200) at 4 $^{\circ}$ C. Biotinylated goat anti-mouse/rabbit IgG (5.0 μ g/ml) were used to incubate the slices for 1 h. Two authors individually took pictures of the immunohistochemically stained slides, and quantitatively analyzed positive staining under a light microscope furnished with a digital camera (ECLIPSE 80i; Nikon, Shinagawa, Tokyo, Japan).

2.8. Statistical analysis

The presentation of all the data are done by way of the mean \pm standard deviation (SD) values, from at least three independent experiments. Using a two-way analysis of variance (ANOVA), followed by Dunnett's post-hoc test, the statistical analysis for comparison between multiple treatment and control groups was performed. Comparison of the differences between the two groups was done using the Student *t*-test. Statistical significance was indicated where *P* values were < 0.05.

3. Results

3.1. Role of Nrf2 in EMT in the mice UUO model

For our study, before we ever used the Nrf2 knockout mice, the total protein from kidney tissues of the WT and Nrf2^{-/-} mice was extracted, and the expression of Nrf2 protein was tested. No expression was observed in Nrf2^{-/-} mice, from the results presented (Supplementary Figs. 1 and 2). All the mice from the four groups survived from the surgery during the mice UUO model, and were forfeited until the planned time points. The complications from surgeries or other

Table 1

Fibrosis score in HE staining among four groups.

Groups	7 days	14 days	21 days
Nrf2 ^{-/-} + Sham	0.20 \pm 0.20	0.20 \pm 0.20	0.40 \pm 0.25
Nrf2 ^{-/-} + UUO	0.80 \pm 0.20	2.20 \pm 0.20*	3.80 \pm 0.20**
WT + Sham	0.20 \pm 0.20	0.40 \pm 0.25	0.60 \pm 0.25
WT + UUO	0.60 \pm 0.25	1.80 \pm 0.20#	3.40 \pm 0.25##

Data are presented as mean \pm SD (*n* = 5 in each group).

* *P* < .001.

** *P* < .0001 compared with Nrf2^{-/-} + Sham.

P < .001.

P < .0001 compared with WT + Sham.

disease resulted in no mice death report. In Fig. 1A, significant renal interstitial fibrosis and tubular atrophy, as well as acute tubular necrosis, were observed in WT + UUO group and Nrf2^{-/-} + UUO group; whereas Masson staining revealed significant renal interstitial fibrosis and accumulation of collagen on day 14 that became more severe on day 21 in the WT + UUO group and Nrf2^{-/-} + UUO group compared to the WT + Sham group and Nrf2^{-/-} + Sham group (Fig. 1B). Furthermore, notable renal fibrosis was witnessed in the Nrf2^{-/-} + UUO group, than with the WT + UUO group (Fig. 1A and B). In Fig. 1C and Table 1, the quantitative analysis of the fibrosis score among the groups is presented.

Concerning the affected proteins, the expression of Nrf2 and E-Cadherin significantly reduced, and the expression of α -SMA significantly improved on day 21 in the WT + UUO group (Fig. 2A–C). Likewise on day 21, the expression of Nrf2 in the WT + UUO group abnormally declined compared to the Sham + WT group (Fig. 2A). The expression of α -SMA in the WT + UUO group considerably increased as compared to the Sham + WT group and Sham + Nrf2^{-/-} group; moreover, the rise was even more noticeable in the Nrf2^{-/-} + UUO group (Fig. 2C). On the other hand, the manifestation of E-cadherin was considerably reduced in the WT + UUO group and Nrf2^{-/-} + UUO group, when compared with the Sham + WT group and Sham + Nrf2^{-/-} group (Fig. 2B).

3.2. Effect of Nrf2 on TGF- β 1-induced EMT in HK-2 cells

In order to test the results witnessed in the UUO models, we treated the HK-2 cell line with TGF- β 1, and analyzed the function of Nrf2 on EMT. Our discovery was that, the expression of Nrf2 was significantly

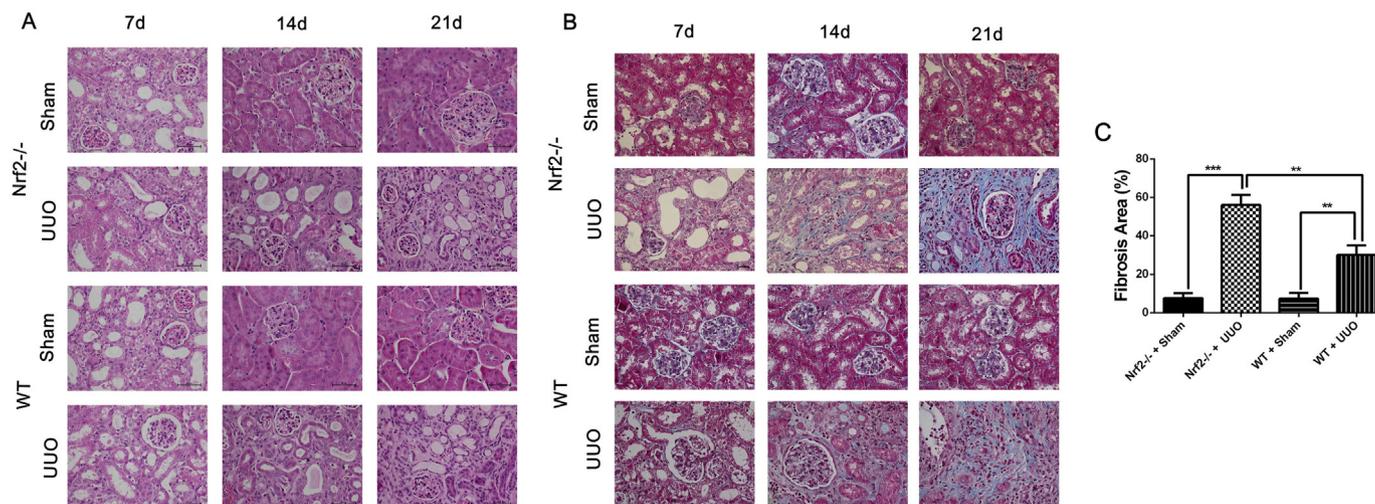


Fig. 1. Results of HE and Masson staining in UUO mice model. Nrf2 knockout mice and wild-type mice were harvested on day 7, 14 and 28. Kidney tissues from these four groups were stained with HE (A) and Masson's trichrome (B) to detect the histopathological changes and collagen accumulation, respectively. The quantitative analysis of Masson staining results was presented as Fig. 1C. **P* < .01, ***P* < .05, ****P* < .001.

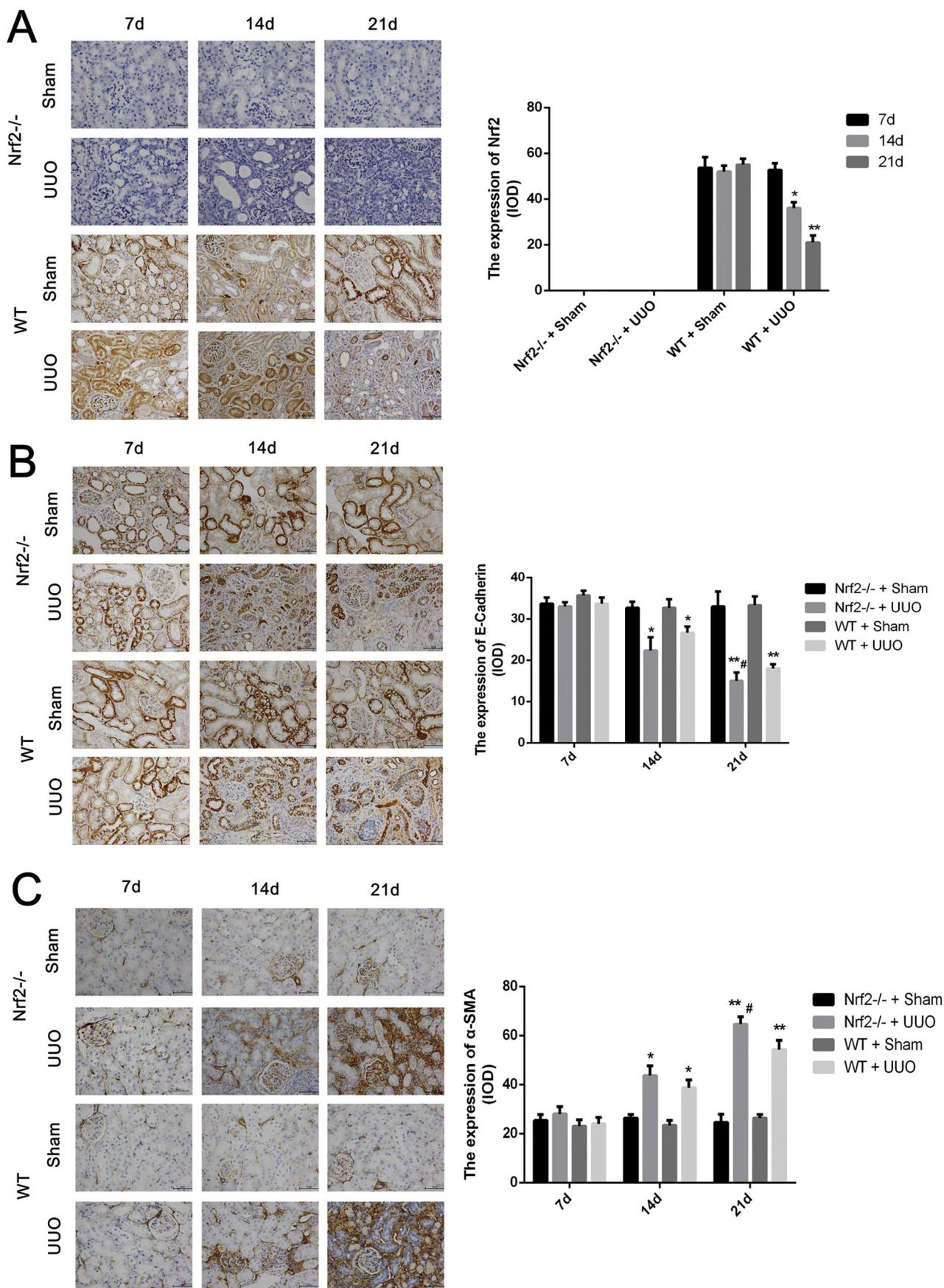


Fig. 2. Relationship between Nrf2 expression and EMT in UUO mice model. Nrf2 knockout mice and wild-type mice were harvested on day 7, 14 and 28. Relative protein expressions of Nrf2 (A), E-Cadherin (B) and α-SMA (C) were examined by immunohistochemical staining. In addition, quantitative analysis of Nrf2, E-Cadherin and α-SMA were also shown in Fig. 2. Data were presented as mean ± SD. **P* < .01, ***P* < .001 compared with 7 days in the same group, # *P* < .05 compared between Nrf2^{-/-} + UUO group and WT + UUO group.

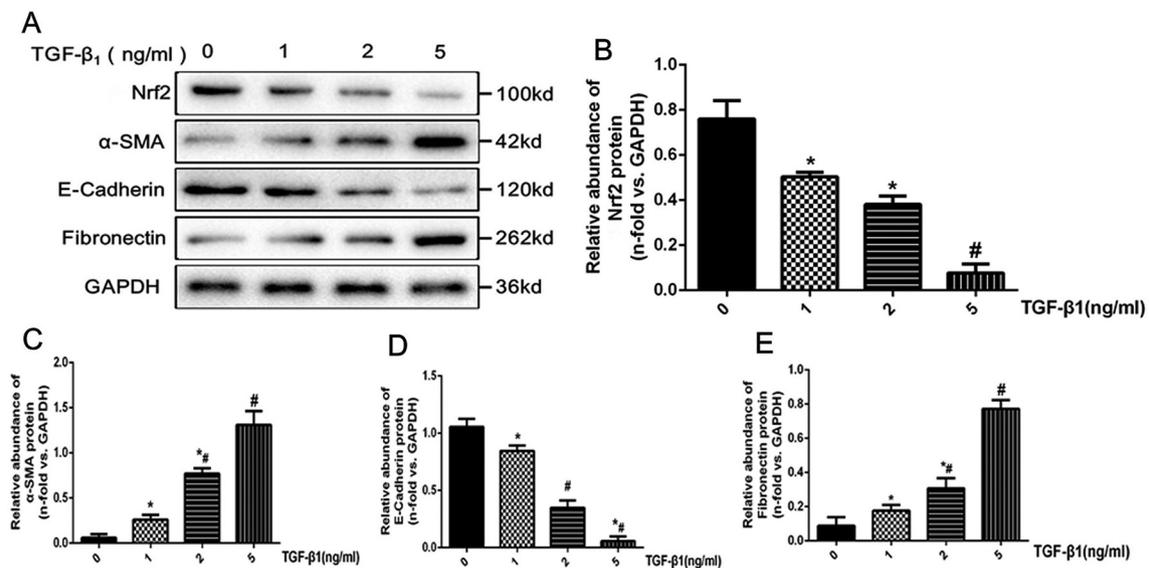


Fig. 3. Relationship between Nrf2 and EMT in HK-2 cells induced by TGF- β 1. HK-2 cells were treated with TGF- β 1 in various concentrations (1–5 ng/ml). Proteins were extracted from cells and western blot assay were performed to assess the expressions of p-Nrf2, E-Cadherin, α -SMA and Fibronectin (A). GAPDH were used as an internal reference for relative quantification of p-Nrf2 (B), E-Cadherin (C), α -SMA (D) and Fibronectin (E). Data were presented as the mean \pm SD, * P < .05 compared with control group; *# P < .001 compared with control group; # P < .0001 compared with control group.

reduced in a dose-dependent method, indicating that Nrf2 may be involved in the suppression of EMT induced by TGF- β 1. Additional, analogous to the *in vivo* results, observed in a dose-dependent manner the up-regulation of α -SMA and Fibronectin expression, as well as down-regulation of E-cadherin expression (Fig. 3A). Fig. 3B–E presents the results of the quantitative analysis.

3.3. Effect of Nrf2 silencing on EMT induced by TGF- β 1 in HK-2 cells

HK-2 cells were transfected with siRNA against Nrf2 so as to determine the effects of Nrf2 silencing on the progress of EMT. At a siRNA concentration of 50 nM, the majority of RNA transfection experiments presented an approximately 80% efficacy of Nrf2 silencing, using the transfection reagent lipofectamine 2000 (Lipo2000). As Fig. 4A illustrates, the expression of E-cadherin was significantly subdued, and the expression of α -SMA proteins was remarkably stimulated in answer to Nrf2 silencing (Fig. 4B–D). Consequently, these results display that, silencing of Nrf2 stimulated the progress of EMT prompted by TGF- β 1.

3.4. Involvement of the PI3K/Akt pathway in Nrf2-induced attenuation of EMT and kidney fibrosis

We treated HK-2 cells with the Nrf2 siRNA and selective inhibitor wortmannin against PI3K, so as to study the signaling pathway involved in the effect of Nrf2 on EMT and kidney fibrosis. Before and after the involvement of siRNA in the protein expressions of p-PI3K and p-Akt, there was no significant difference found (Fig. 4A). Compared to the application of only TGF- β , 1, the application of wortmannin along with TGF- β 1 significantly improved the expression of the p-Nrf2 protein, as presented in Fig. 4E. Contrarily, the expression of α -SMA abnormally dropped, just like the expression of phosphorylated PI3K and its phosphorylated downstream modulator Akt (Fig. 4E). The PI3K/Akt/Nrf2 signaling pathway might be implicated in the progress of EMT, from the indications of these findings. Additionally, from the UUO models, we examined the expression of the PI3K/Akt signaling pathway molecules, in the kidney tissue harvested. Parallel to the *in vitro* verdicts, there was a meaningful increase in the expression of phosphorylated PI3K and Akt in the Nrf2^{-/-} + UUO group, as compared to the WT + UUO group (Fig. 5A). These findings indicate that, through the PI3K/Akt signaling pathway, Nrf2 significantly reduced the progress of EMT and renal

interstitial fibrosis.

4. Discussion

In this current study, through the PI3K/Akt signaling pathway in a UUO model of Nrf2^{-/-} mice and HK-2 cells, we studied the function of Nrf2 as an antifibrotic modulator in the development of EMT and kidney fibrosis.

Latest studies have revealed that Nrf2 principally functions as an antifibrotic modulator in regulating anti-inflammatory and nitric oxide reactions, taking part in the pathogenesis of kidney fibrosis (Arellano-Buendia et al., 2016; Qin et al., 2016; Kim et al., 2015). Furthermore, collected proof is available to support the important relationship between the Nrf2 signaling pathway and EMT progress in kidney fibrosis. Like for instance, it was reported that, epigallocatechin-3-gallate exerts a protective effect against acute renal damage through its anti-oxidative effect, by means of the activation of the Nrf2 signaling pathway (Wang et al., 2015). Additionally, the protective consequences of Nrf2 modulation on kidney fibrosis were also witnessed in the streptozotocin-induced diabetic mouse model (Zhang et al., 2016). Likewise, curcumin was recounted to shield renal tubular epithelial cells from high glucose-induced EMT, by means of the Nrf2-mediated upregulation of HO-1 (Zhang et al., 2015). Also, in this current study, we recognized a solid relationship between Nrf2 expression and EMT development. We discovered that as a modulator, Nrf2 could act as a protector in EMT and kidney interstitial fibrosis, for the UUO model of Nrf2^{-/-} mice. All in all these observations entail that, there is a reduced Nrf2 expression in the pathogenesis of kidney fibrosis and chronic kidney diseases. Consequently, Nrf2 activation may present a new healing strategy to prevent kidney interstitial fibrosis.

Concerning the mechanisms taking part in the antifibrotic effects of Nrf2 signaling pathways, like the TGF β /Smad pathway, Nrf2/ARE pathway and MAPK pathway, they have been involved in the launch and development of tissue fibrosis (Qin et al., 2016; Yang et al., 2017; Xu and Kong, 2017; Chen et al., 2017). TGF- β 1 is known to induce the surplus production of ECM in renal tubular epithelial cells, and the considerable buildup of ECM in the renal interstitium, as well as the development of EMT, and that it facilitates its profibrotic effects through Smad-dependent pathways (Sutariya et al., 2016; Ha and Lee, 2003). The substantial relationship between the inhibition of the TGF β /

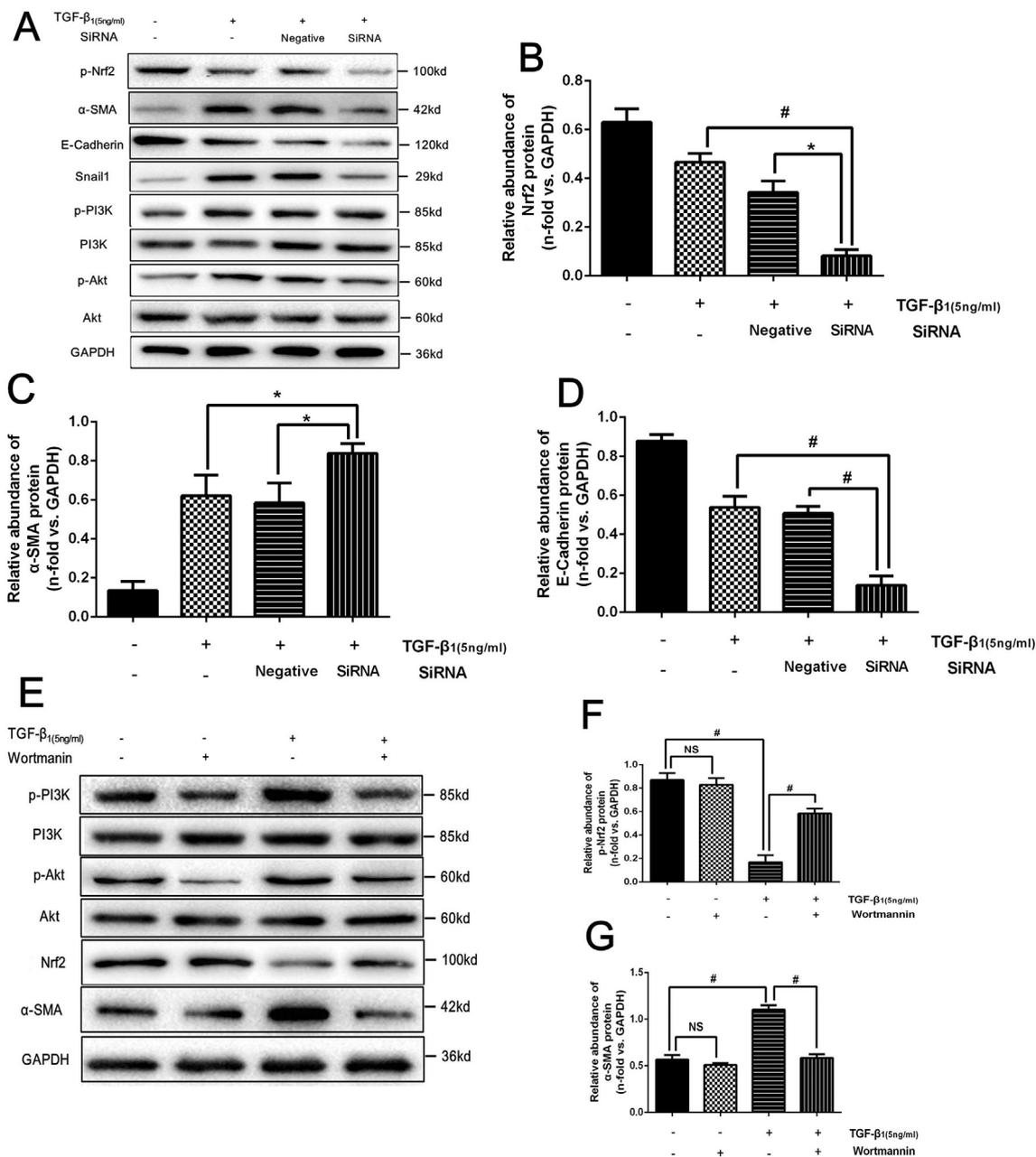


Fig. 4. Nrf2-related signaling in the EMT in HK-2 cells induced by TGF- β 1. Nrf2 siRNA and pCMV-Nrf2 plasmid were transfected into HK-2 cells treated with TGF- β 1 (5 ng/ml), respectively. Negative RNA or plasmid was used as control. Expression of Nrf2, Keap1, HO-1 and NQO1 proteins were examined by Western blot assay (Fig. 4A, F). Quantitative analysis of Nrf2 (B, G), Keap1 (C, H), HO-1 (D, I) and NQO1 (E, J) were performed. Data were presented as the mean \pm SD, * P < .05 compared with intervention group.

Smad pathway with Nrf2-regulated damage in tissue fibrosis, is indicated by the increasing evidence (Wang et al., 2015; Zhang et al., 2016; Oh et al., 2012). Nrf2 functions as a sensor of oxidative or electrophilic stress, since it's an important modulator of antioxidant activity, and averts genome instability (Geismann et al., 2014). Nrf2 cytoprotective activity has served in to prevent various diseases, particularly hindering cancer's development initial stage (Krajka-Kuzniak et al., 2016). The canonical pathway is in charge of the main activation of Nrf2, which interrelates with its cytosolic repressor protein Keap1 to form the Nrf2-Keap1 complex, clarifying the opposite expression of Nrf2 and Keap1 in the cell culture. This leads to the upregulation of Nrf2-targeted genes and the conscription of vital factors for transcription facilitated by AREs (Kang and Hyun, 2017). Therefore, the Nrf2/ARE signaling pathway remains an indispensable therapeutic target of cancer and further Nrf2-related diseases. Additionally, the p38 MAPK/

Nrf2 signaling pathway was equally defined as a probable target for the induction of HO-1 and NQO1 in adipocytes (Wang et al., 2017). During our study, the PI3K/Akt signaling pathway was proven to be indispensable to prevent EMT and kidney fibrosis by Nrf2 signaling (Nrf2/Keap1/HO-1/NQO1). Studies on the cytoprotective effects of chlorogenic acid and protective effect of melatonin in liver injury are consistent with our results, they equally involve the PI3K/Akt pathway in the hindrance of EMT and fibrosis (Zhang et al., 2017; Han et al., 2017).

To conclude, our findings demonstrate that Nrf2 signaling plays a significant role EMT development and renal interstitial fibrosis, modulated by the PI3K/Akt signaling pathway. A new insight is brought by our findings, concerning the prevention and treatment of kidney fibrosis in several chronic kidney diseases.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yexmp.2019.104296>.

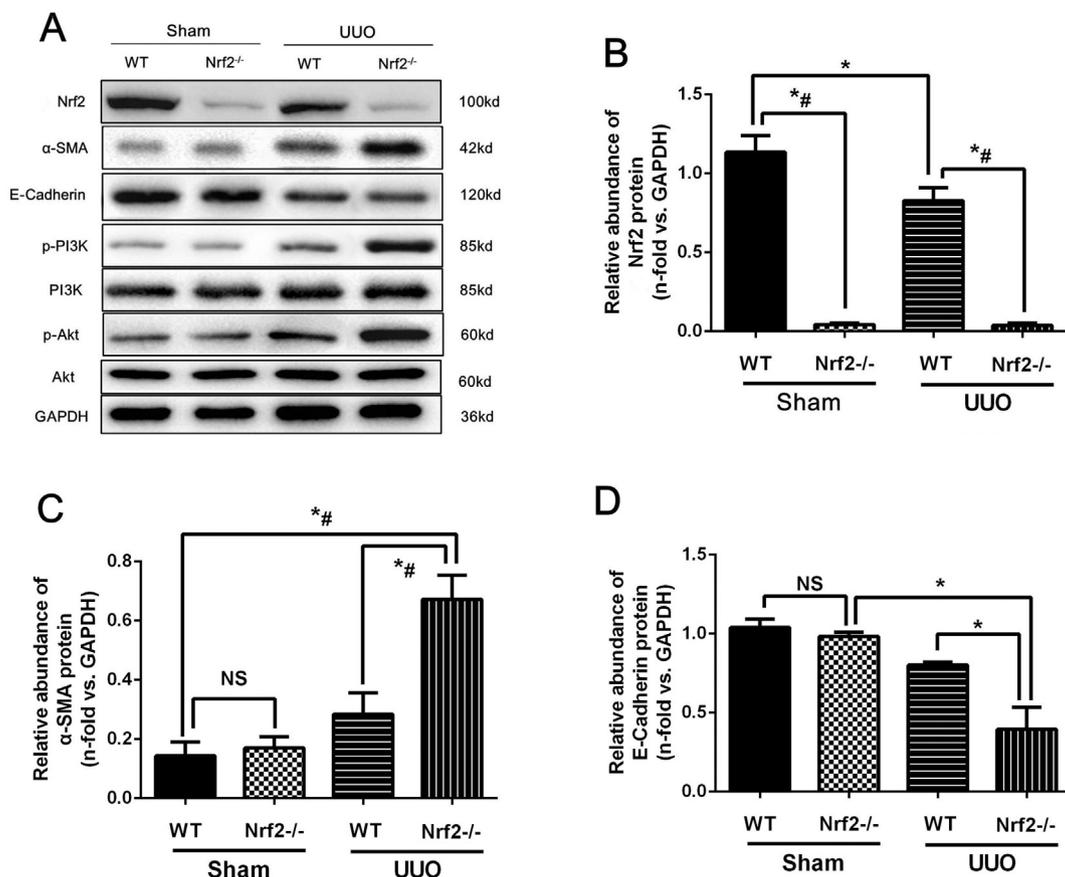


Fig. 5. Pathways investigation in HK-2 cells induced by TGF-β1. Nrf2 siRNA were transfected in HK-2 cells treated with TGF-β1 (5 ng/ml), and expressions of Nrf2, E-Cadherin and α-SMA were assessed by western blot assay (A). Quantitative analysis of Nrf2 (B), E-Cadherin (C) and α-SMA (D) were performed. Furthermore, selective inhibitor of PI3K were used to explore the pathways involved in the HK-2 cells treated with TGF-β1 (5 ng/ml) (E). Quantitative analysis of Nrf2 (F) and α-SMA (G) were performed. Data were presented as the mean ± SD, *P < .05 compared with control group; #P < .0001 compared with control group.

Declaration of Competing Interest

The authors have declared that no competing interests exist.

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References

Arellano-Buendia, A.S., Tostado-Gonzalez, M., Garcia-Arroyo, F.E., Cristobal-Garcia, M., Loredano-Mendoza, M.L., Tapia, E., et al., 2016. Anti-inflammatory therapy modulates Nrf2-Keap1 in kidney from rats with diabetes. *Oxidative Med. Cell. Longev.* 2016 (4693801).

Chen, Z., Xie, X., Huang, J., Gong, W., Zhu, X., Chen, Q., et al., 2017. Connexin43 regulates high glucose-induced expression of fibronectin, ICAM-1 and TGF-beta1 via Nrf2/ARE pathway in glomerular mesangial cells. *Free Radic. Biol. Med.* 102, 77–86.

Geismann, C., Arlt, A., Sebens, S., Schafer, H., 2014. Cytoprotection "gone astray": Nrf2 and its role in cancer. *Onco Targets Ther.* 7, 1497–1518.

Ha, H., Lee, H.B., 2003. Reactive oxygen species and matrix remodeling in diabetic kidney. *J. Am. Soc. Nephrol.* 14 (8 Suppl 3), S246–S249.

Han, D., Chen, W., Gu, X., Shan, R., Zou, J., Liu, G., et al., 2017. Cytoprotective effect of chlorogenic acid against hydrogen peroxide-induced oxidative stress in MC3T3-E1 cells through PI3K/Akt-mediated Nrf2/HO-1 signaling pathway. *Oncotarget.* 8 (9), 14680–14692 2017 Feb 28.

Hay, E.D., 1995. An overview of epithelio-mesenchymal transformation. *Acta Anat.* 154 (1), 8–20.

He, D., Wang, S., Jia, Z., Cui, L., Lu, Y., Hu, H., et al., 2015. Calcium ions promote primary renal epithelial cell differentiation into cells with bone-associated phenotypes via transforming growth factor-beta1-induced epithelial-mesenchymal transition in idiopathic hypercalcaemia patients. *Mol. Med. Rep.* 11 (3), 2199–2206.

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J.D., et al., 1999. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through

binding to the amino-terminal Neh2 domain. *Genes Dev.* 13 (1), 76–86.

Iwano, M., Plieth, D., Danoff, T.M., Xue, C., Okada, H., Neilson, E.G., 2002. Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J. Clin. Invest.* 110 (3), 341–350.

Kalluri, R., Neilson, E.G., 2003. Epithelial-mesenchymal transition and its implications for fibrosis. *J. Clin. Invest.* 112 (12), 1776–1784.

Kang, K.A., Hyun, J.W., 2017. Oxidative stress, Nrf2, and epigenetic modification contribute to anticancer drug resistance. *Toxicol. Res.* 33 (1), 1–5.

Kay, H.Y., Kim, Y.W., Ryu, D.H., Sung, S.H., Hwang, S.J., Kim, S.G., 2011. Nrf2-mediated liver protection by saquinone, an antioxidant lignan, from acetaminophen toxicity through the PKCdelta-GSK3beta pathway. *Br. J. Pharmacol.* 163 (8), 1653–1665.

Kim, S., Kim, S.J., Yoon, H.E., Chung, S., Choi, B.S., Park, C.W., et al., 2015. Fimasartan, a novel angiotensin-receptor blocker, protects against renal inflammation and fibrosis in mice with unilateral ureteral obstruction: the possible role of Nrf2. *Int. J. Med. Sci.* 12 (11), 891–904.

Krajka-Kuzniak, V., Paluszczak, J., Baer-Dubowska, W., 2016. The Nrf2-ARE signaling pathway: an update on its regulation and possible role in cancer prevention and treatment. *Pharmacol. Rep.* 69 (3), 393–402.

Landolt, L., Eikrem, O., Strauss, P., Scherer, A., Lovett, D.H., Beisland, C., et al., 2017. Clear cell renal cell carcinoma is linked to epithelial-to-mesenchymal transition and to fibrosis. *Phys. Rep.* 5 (11).

Liu, Y., 2004. Epithelial to mesenchymal transition in renal fibrogenesis: pathologic significance, molecular mechanism, and therapeutic intervention. *J. Am. Soc. Nephrol.* 15 (1), 1–12.

Lopez-Guisa, J.M., Rassa, A.C., Cai, X., Collins, S.J., Eddy, A.A., 2011. Vitronectin accumulates in the interstitium but minimally impacts fibrogenesis in experimental chronic kidney disease. *Am. J. Physiol. Ren. Physiol.* 300 (5), F1244–F1254.

Ma, Q., 2013. Role of nrf2 in oxidative stress and toxicity. *Annu. Rev. Pharmacol. Toxicol.* 53, 401–426.

Ma, Q., Battelli, L., Hubbs, A.F., 2006. Multiorgan autoimmune inflammation, enhanced lymphoproliferation, and impaired homeostasis of reactive oxygen species in mice lacking the antioxidant-activated transcription factor Nrf2. *Am. J. Pathol.* 168 (6), 1960–1974.

Marcucci, F., Stassi, G., De Maria, R., 2016. Epithelial-mesenchymal transition: a new target in anticancer drug discovery. *Nat. Rev. Drug Discov.* 15 (5), 311–325.

Oh, C.J., Kim, J.Y., Choi, Y.K., Kim, H.J., Jeong, J.Y., Bae, K.H., et al., 2012. Dimethylfumarate attenuates renal fibrosis via NF-E2-related factor 2-mediated inhibition of transforming growth factor-beta/Smad signaling. *PLoS ONE* 7 (10),

- e45870.
- Okada, H., Inoue, T., Suzuki, H., Strutz, F., Neilson, E.G., 2000. Epithelial-mesenchymal transformation of renal tubular epithelial cells in vitro and in vivo. *Nephrol. Dial. Transplant.* 15 (Suppl. 6), 44–46.
- Qin, T., Yin, S., Yang, J., Zhang, Q., Liu, Y., Huang, F., et al., 2016. Sinomenine attenuates renal fibrosis through Nrf2-mediated inhibition of oxidative stress and TGFbeta signaling. *Toxicol. Appl. Pharmacol.* 304, 1–8.
- Risdon, R.A., Sloper, J.C., De Wardener, H.E., 1968. Relationship between renal function and histological changes found in renal-biopsy specimens from patients with persistent glomerular nephritis. *Lancet.* 2 (7564), 363–366.
- Ryoo, I.G., Ha, H., Kwak, M.K., 2014. Inhibitory role of the KEAP1-NRF2 pathway in TGFbeta1-stimulated renal epithelial transition to fibroblastic cells: a modulatory effect on SMAD signaling. *PLoS ONE* 9 (4), e93265.
- Ryoo, I.G., Shin, D.H., Kang, K.S., Kwak, M.K., 2015. Involvement of Nrf2-GSH signaling in TGFbeta1-stimulated epithelial-to-mesenchymal transition changes in rat renal tubular cells. *Arch. Pharm. Res.* 38 (2), 272–281.
- Sakai, N., Chun, J., Duffield, J.S., Lagares, D., Wada, T., Luster, A.D., et al., 2017. Lysophosphatidic acid signaling through its receptor initiates profibrotic epithelial cell fibroblast communication mediated by epithelial cell derived connective tissue growth factor. *Kidney Int.* 91 (3), 628–641.
- Shin, D.H., Park, H.M., Jung, K.A., Choi, H.G., Kim, J.A., Kim, D.D., et al., 2010. The NRF2-heme oxygenase-1 system modulates cyclosporin A-induced epithelial-mesenchymal transition and renal fibrosis. *Free Radic. Biol. Med.* 48 (8), 1051–1063.
- Soranno, D.E., Lu, H.D., Weber, H.M., Rai, R., Burdick, J.A., 2014. Immunotherapy with injectable hydrogels to treat obstructive nephropathy. *J. Biomed. Mater. Res. A* 102 (7), 2173–2180.
- Sutariya, B., Jhonsa, D., Saraf, M.N., 2016. TGF-beta: the connecting link between nephropathy and fibrosis. *Immunopharmacol. Immunotoxicol.* 38 (1), 39–49.
- Truong, L.D., Petrussevska, G., Yang, G., Gurpinar, T., Shappell, S., Lechago, J., et al., 1996. Cell apoptosis and proliferation in experimental chronic obstructive uropathy. *Kidney Int.* 50 (1), 200–207.
- Tsai, P.Y., Ka, S.M., Chao, T.K., Chang, J.M., Lin, S.H., Li, C.Y., et al., 2011. Antroquinonol reduces oxidative stress by enhancing the Nrf2 signaling pathway and inhibits inflammation and sclerosis in focal segmental glomerulosclerosis mice. *Free Radic. Biol. Med.* 50 (11), 1503–1516.
- Wang, Y., Liu, N., Su, X., Zhou, G., Sun, G., Du, F., et al., 2015. Epigallocatechin-3-gallate attenuates transforming growth factor-beta1 induced epithelial-mesenchymal transition via Nrf2 regulation in renal tubular epithelial cells. *Biomed. Pharmacother.* 70, 260–267.
- Wang, Z., Han, Z., Tao, J., Wang, J., Liu, X., Zhou, W., et al., 2016. Transforming growth factor-beta1 induces endothelial-to-mesenchymal transition via Akt signaling pathway in renal transplant recipients with chronic allograft dysfunction. *Ann. Transplant.* 21, 775–783.
- Wang, Z., Ka, S.O., Lee, Y., Park, B.H., Bae, E.J., 2017. Butein induction of HO-1 by p38 MAPK/Nrf2 pathway in adipocytes attenuates high-fat diet induced adipose hypertrophy in mice. *Eur. J. Pharmacol.* 799, 201–210.
- Xu, Z., Kong, X.Q., 2017. Bixin ameliorates high fat diet-induced cardiac injury in mice through inflammation and oxidative stress suppression. *Biomed. Pharmacother.* 89, 991–1004.
- Yang, N., Shi, J.J., Wu, F.P., Li, M., Zhang, X., Li, Y.P., et al., 2017. Caffeic acid phenethyl ester up-regulates antioxidant levels in hepatic stellate cell line T6 via an Nrf2-mediated mitogen activated protein kinases pathway. *World J. Gastroenterol.* 23 (7), 1203–1214.
- Yu, Z., Strutz, J.M., Kipnis, V., White, S.N., 1995. Effect of dynamic loading methods on cement film thickness in vitro. *J. Prosthodont.* 4 (4), 251–255.
- Zeisberg, M., Bonner, G., Maeshima, Y., Colorado, P., Muller, G.A., Strutz, F., et al., 2001. Renal fibrosis: collagen composition and assembly regulates epithelial-mesenchymal transdifferentiation. *Am. J. Pathol.* 159 (4), 1313–1321.
- Zeisberg, E.M., Potenta, S.E., Sugimoto, H., Zeisberg, M., Kalluri, R., 2008. Fibroblasts in kidney fibrosis emerge via endothelial-to-mesenchymal transition. *J. Am. Soc. Nephrol.* 19 (12), 2282–2287.
- Zhang, X., Liang, D., Guo, L., Liang, W., Jiang, Y., Li, H., et al., 2015. Curcumin protects renal tubular epithelial cells from high glucose-induced epithelial-to-mesenchymal transition through Nrf2-mediated upregulation of heme oxygenase-1. *Mol. Med. Rep.* 12 (1), 1347–1355.
- Zhang, X., He, H., Liang, D., Jiang, Y., Liang, W., Chi, Z.H., et al., 2016. Protective effects of berberine on renal injury in streptozotocin (STZ)-induced diabetic mice. *Int. J. Mol. Sci.* 17 (8).
- Zhang, Y., Wei, Z., Liu, W., Wang, J., He, X., Huang, H., et al., 2017. Melatonin protects against arsenic trioxide-induced liver injury by the upregulation of Nrf2 expression through the activation of PI3K/AKT pathway. *Oncotarget.* 8 (3), 3773–3780.
- Zou, X.F., Gu, J.H., Cui, Z.L., Lu, Y.W., Gu, C., 2017. CXCR4 chemokine receptor type 4 antagonism ameliorated allograft fibrosis in rat kidney transplant model. *Exp. Clin. Transplant.* 15 (4), 448–452 2017 Aug.