ORIGINAL RESEARCH

Emodin Retarded Renal Fibrosis Through Regulating HGF and TGFβ–Smad Signaling Pathway

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Background: Renal fibrosis is a frequently occurring type of chronic kidney disease that can cause end-stage renal disease. It has been verified that emodin or HGF can inhibit the development of renal fibrosis. However, the antifibrotic effect of emodin in combination with HGF remains unclear.

Methods: Cell viability was detected with CCK8. Gene and protein expression in HK2 cells was detected by qRT-PCR and Western blot, respectively. Moreover, a unilateral ureteral obstruction–induced mouse model of renal fibrosis was established for investigating the antifibrotic effect of emodin in combination with HGF in vivo.

Results: HGF notably increased the expression of collagen II in TGFβ-treated HK2 cells. In addition, HGF-induced increase in collagen II expression was further enhanced by emodin. In contrast, fibronectin, αSMA and Smad2 expression in TGFβ-stimulated HK2 cells was significantly inhibited by HGF and further decreased by combination treatment (emodin plus HGF). Moreover, we found that combination treatment exhibited better antifibrotic effects compared with emodin or HGF in vivo.

Conclusion: These data demonstrated that emodin plus HGF exhibited better antifibrotic effects compared with emodin or HGF. As such, emodin in combination with HGF may serve as a new possibilty for treatment of renal fibrosis.

Keywords: fibrosis, combination, emodin, TGFβ

Introduction

Renal fibrosis is considered to originate from activation of myofibroblasts in the kidney and infiltration of inflammatory cells.[1,2] A number of patients with chronic kidney disease will deteriorate to end-stage renal disease (ESRD) once renal fibrosis occurs. The only effective method is transplantation in that situation.[3] Furthermore, it has been confirmed that chronic kidney disease patients account for 10% of the world's total population.[4,5] As such, a new method for cure of renal fibrosis is of great significance.

HGF) contains an α-chain and a β-chain. These chains are composed of a serine protease–like structure and four kringle domains.[6,7] Upregulation of the HGF–Met pathway initates dynamic biological responses that help morphogenesis (eg, epithelial tubulogenesis) and cells survival.[8] Recent reports have indicated that HGF has a significant antifibrotic effect.[9,10] In addition, *Rheum officinale* has been confirmed to have some bioactivity, including antifibrotic activity.[11,12] Emodin is the major bioactive compound of *R. officinale* and has strong anti-

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inflammatory and antioxidant properties.[13] It has been reported that emodin notably suppresses the development of multiple diseases.[14,15] Additionally, it has previously been verified that emodin can relieve the progression of renal fibrosis.[16] However, the antifibrotic effect of emodin in combination with HGF has not been confirmed yet. TGFβ participates in many cellular processes, including cell growth, differentiation, apoptosis, and homeostasis.[17,18] Many studies have indicated that TGFβ plays a crucial role in renal fibrosis.[19,20] Yasumura et al verified that TGFβ contributes to the occurrence of renal fibrosis.[21] In this study, we sought to confirm whether emodin can increase the antifibrotic effect of HGF, with the purpose of providing new strategies for patients.

Methods
Cell Culture

Human renal tubular endothelial cells (HK2; ATCC, Manassas, VA, USA) were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS and 1% penicillin (Thermo Fisher Scientific) in an incubator (37°C, 5% CO_2). To mimic renal fibrosis in vitro, HK2 cells were stimulated with $TGFβ_1$ (5 ng/mL; PeproTech, Rocky Hill, NJ, USA) for 48 hours.

CCK8 Assays

HK2 cells (5×10^3 cells/well) were seeded at 37°C overnight. Then, cells were treated with emodin (10, 20, 40, and 60 μM) for 2 days. After that, 10 μL CCK8 reagent (Beyotime) per well was added for 2 hours. Finally, cell viability was measured with a microplate reader (Bio-Rad Laboratories, Benicia, CA, USA) at 450 nm absorbance. Standard emodin products (95% purity) were purchased from Sigma-Aldrich (St Louis, MO, USA).

Immunofluorescence Staining

HK2 cells were seeded overnight. After 72 hours' treatment, cells were blocked with 10% FBS for 30 minutes and then incubated with antiSmad4 antibody (Abcam, Cambridge, MA, USA; 1:1,000) overnight. Then, cells were incubated with goat antirabbit IgG (Abcam; 1:5,000) for 1 hour. After that, the nuclei were stained with DAPI (Beyotime) for 5 minutes. Finally, cells were observed under microscopy (Olympus CX23, Tokyo, Japan).

Reverse-Transcription Quantitative Polymerase Chain Reaction

Total RNA was extracted using Trizol reagent (Thermo Fisher Scientific). RNA integrity was measured by agarose-gel electrophoresis. Then, cDNA was obtained by reverse transcription (PrimeScript first-strand cDNA-synthesis kit; Takara, Tokyo, Japan). PCR reactions were carried out by SYBR Premix Ex Taq II (Takara) with an Applied Biosystems 7500 real-time PCR system(Thermo Fisher Scientific). Primers were: collagen II — forward 5′-AACCAAGGGAAGCATTACTAC-3′, reverse 5′-GAGCAAACTATTCCATAAGCC-3′; fibronectin — forward 5′-CTTGAAGCCCAGCAACCTAC-3′, reverse 5′-ACAGCCCACAGACCACACAT-3′; αSMA — forward 5′-TTTGAGACGAGGGAGATGGT-3′, reverse 5′-GTTCTTGGTTGGAGCGGAT-3′; and GAPDH — forward 5′-GTCCACCGCAAATGCTTCTA-3′, reverse 5′-TGCTGTCACCTTCACCGTTC-3′. Amplification conditions were 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 34 seconds. The $2^{-\Delta\Delta Ct}$ method was used to quantify the data. β-actin was used as an internal control.

Western Blot

Cells or renal tissue were lysed using RIPA lysis buffer (Beyotime). Protein concentration was quantified using a BCA protein-assay kit (Beyotime). Protein (30 μg) was separated with 10% SDS-PAGE and then transferred into PVDF membranes (Beyotime). After blocking with 5% skim milk, the membranes were incubated with primary antibodies (anti-collagen II 1:1,000, anti-αSMA 1:1,000; anti-fibronectin 1:1,000; anti-Smad2 1:1,000; and anti-GAPDH 1:1,000) at 4°C overnight. All primary antibodies were purchased from Abcam. Then, membranes were incubated with secondary antibodies (goat antirabbit IgG; Abcam, 1:5,000) at room temperature for 1 hour. Protein bands were visualized using BeyoECL Plus (Beyotime) and measured using IPP Image-Pro Plus software.

Animal Study

The experimental protocol was approved by the Ethics Committee for Animal Experimentation of Southwest Medical University, and animal study was performed according to the Guidelines for Animal Experimentation of Southwest Medical University. The ethical approval reference number of the study is 20191010.C57BL/6 SPF class–bred mice (18±2g) were obtained from the
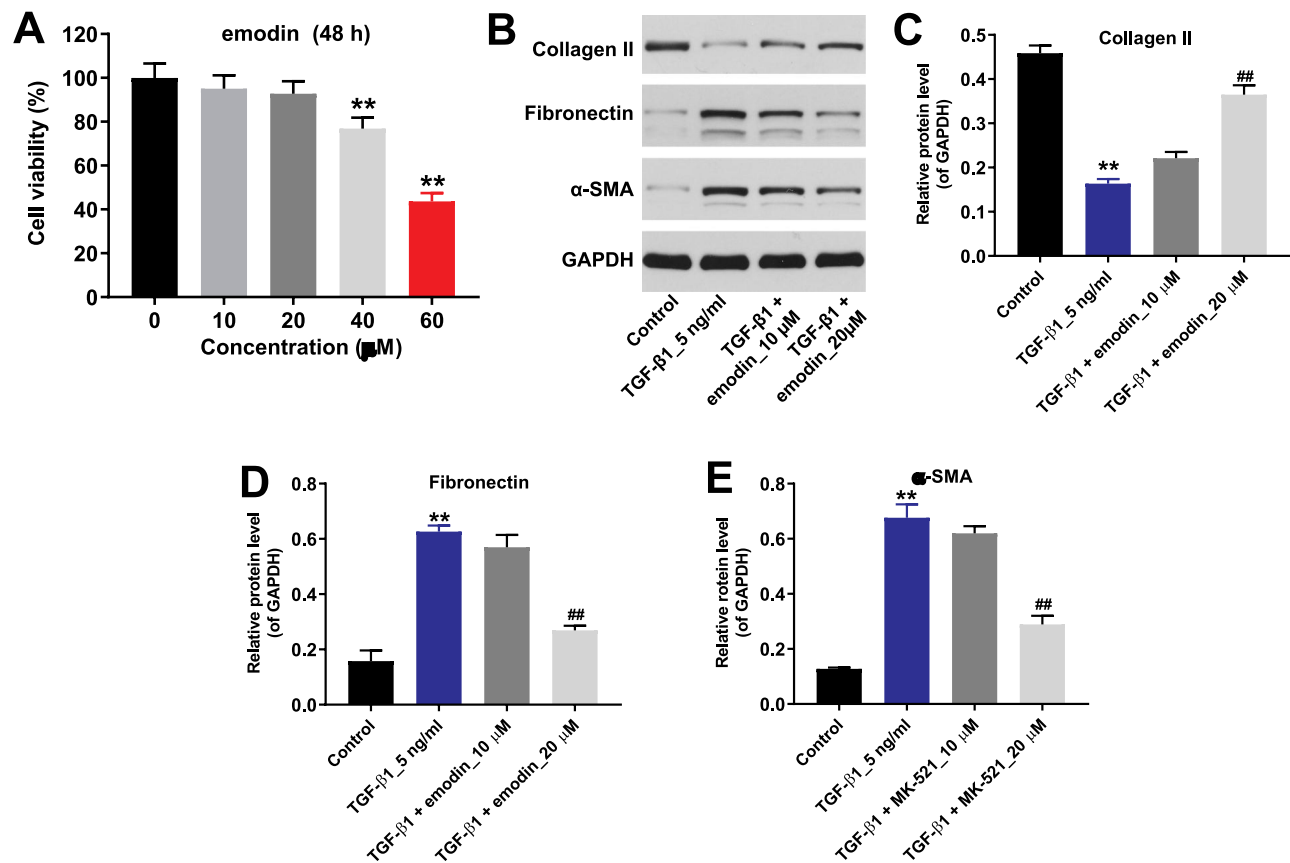
Figure 1 Emodin significantly suppressed the fibrotic effect of TGFβ₁ in vitro. (**A**) HK2 cells were treated with 0, 10, 20, 40, and 60 μM emodin for 48 hours and cell viability determined with CCK8 assay. (**B**) HK2 cells were treated with 5 ng/mL TGFβ₁, 5 ng/mL TGFβ₁ + 10 μM emodin, or 5 ng/mL TGFβ₁ + 20 μM emodin for 48 hours. Then, collagen II, fibronectin, and αSMA expression in HK2 cells was detected by Western blot. (**C**) Relative expression of collagen II was quantified normalized to GAPDH. (**D**) Relative expression of fibronectin was quantified normalized to GAPDH. (**E**) Relative expression of αSMA was quantified normalized to GAPDH. **$P<0.01$ vs 0 μM group or control; ##$P<0.01$ vs TGFβ₁ group.

Laboratory Animal Center of Southwest Medical University with. Mice were housed in a 12-hour light/12-hour dark cycle at 22°C–24°C. After 1 week of adaptive feeding, mice were divided into groups for the following 3 weeks: control (n=12, treated with normal saline), unilateral ureteral obstruction (UUO; n=12, treated with normal saline) UUO plus 50 mg/kg/day emodin (n=12, orally treated with 50 mg/kg/day emodin, emodin dissolved in 2 mL normal saline), UUO plus 0.5 μg/kg/day HGF (n=12, intravenous injection of 0.5 μg/kg/day HGF, HGF dissolved in 1 mL normal saline), and UUO+ HGF + emodin (n=12, injection of 0.5 μg/kg/day HGF and orally treated with 50 mg/kg/day emodin). Standard HGF was purchased from MedChemExpress (99.36% purity, Monmouth Junction, NJ, USA). The dosages of HGF and emodin were selected based on previous research.[22,23] After treatment, the level of creatinine (Cr) and blood urea nitrogen (BUN) were examined. Kidney tissuewas extracted from each mouse and

dissected, then symptoms of renal fibrosis was detected with H&E and Masson's trichrome. The severity of fibrosis was assessed by calculating the ratio of blue-stained scarred areas to total area.

Enzyme-Linked Immunosorbent Assays

At the end of the animal study, mice were killed for collection of sera and levels of Cr and BUN measured with an ELISA kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Statistical Analysis

For each group, at least three independent experiments were performed. All data are expressed as means ± SD. Comparisons among multiple groups were analyzed using one-way ANOVA followed by Tukey's test. $P<0.05$ was considered statistically significant.
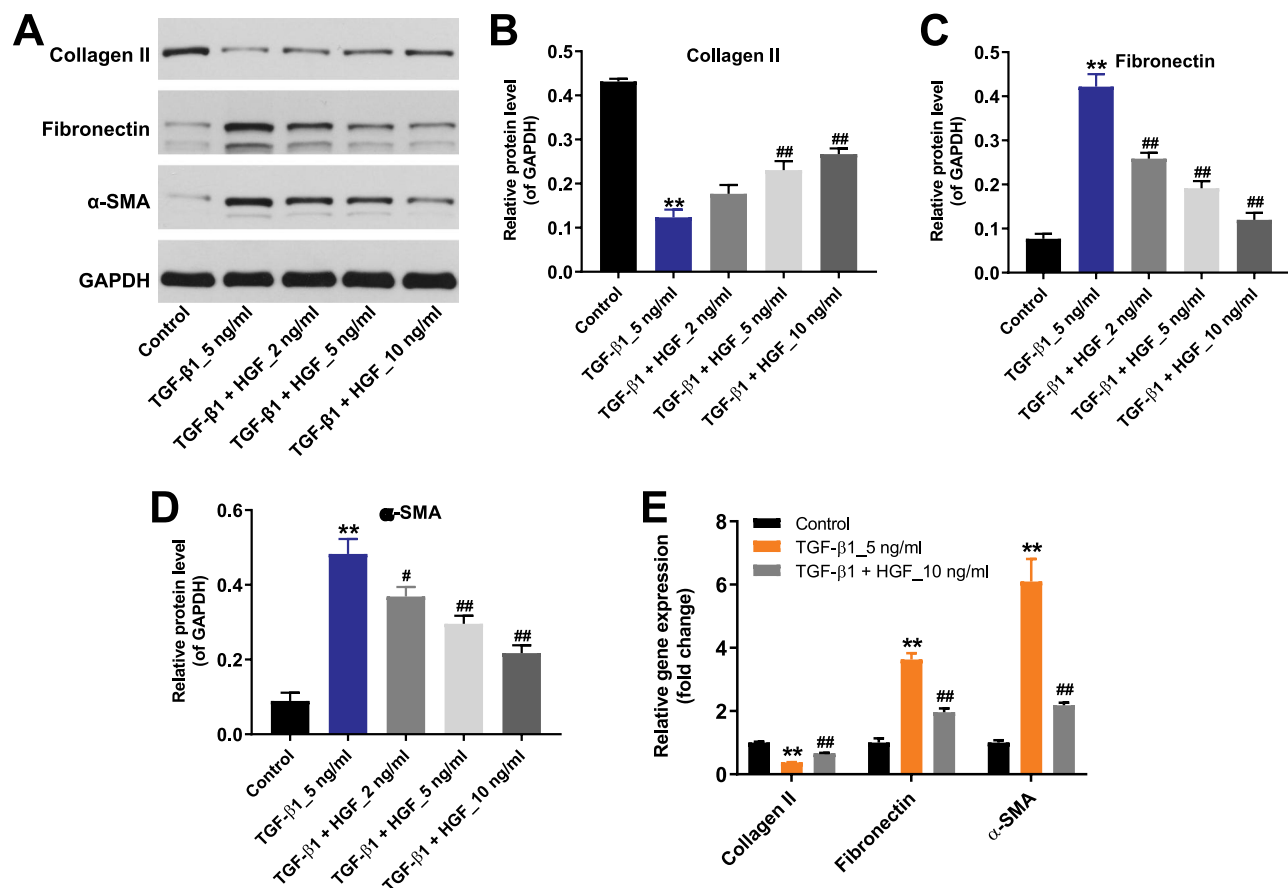
Figure 2 HGF significantly decreased the fibrotic effect of TGFβ₁ in HK2 cells. HK2 cells were treated with 5 ng/mL TGFβ₁ and then with 2, 5 or 10 ng/mL HGF for 48 hours. Then, expression of (**A** and **B**) collagen II, (**A** and **C**) fibronectin and αSMA, and (**A** and **D**) in HK2 cells was detected by Western blot. Relative protein expression was quantified normalized to GAPDH (**E**). Collagen II, fibronectin, and αSMA expression in HK2 cells was detected by qPCR. GAPDH was used as an internal control. **P < 0.01 vs control; #P < 0.05 vs TGFβ₁ group; ##P < 0.01 vs TGFβ₁.

Results

Emodin Significantly Suppressed Fibrotic Effect of TGFβ₁ In Vitro

For detection of the cytotoxicity of emodin on HK2 cells, CCK8 assays were used. As shown in Figure 1A, compared to 0 μM, 40 or 60 μM emodin significantly inhibited cell viability, while 10 or 20 μM had no significant effect. These data demonstrated that ≥40 μM emodin showed obvious cytotoxicity. Furthermore, as indicated in Figure 1B–E, the expression of collagen II in HK2 cells was notably suppressed by TGFβ₁, which was significantly rescued by 20 μM emodin. In contrast, TGFβ₁ markedly activated fibronectin and αSMA in HK2 cells, while TGFβ₁-induced increase of these two proteins was significantly reversed by 20 μM emodin. As we know, fibronectin, collagen II, and αSMA are the key markers in fibrosis.[24,25] Emodin 10 μM affected collagen II, fibronectin, and αSMA expression to a limited. Therefore, 20 μM emodin was selected for the following experiments. These data showed that emodin significantly suppressed the fibrotic effect of TGFβ₁ in vitro.

HGF Significantly Decreased Fibrotic Effect of TGFβ₁ in HK2 Cells

To detect protein expression, Western blot was used. As demonstrated in Figure 2A–D, TGFβ₁ obviously upregulated fibronectin and αSMA and downregulated collagen II in HK2 cells. Furthermore, 2, 5, or 10 ng/mL HGF dose-dependently activated collagen II and suppressed protein levels of fibronectin and αSMA in HK2 cells after TGFβ₁ treatment. Since 10 ng/mL HGF exhibited a better antifibrotic effect compared with the other two concentrations, 10 ng/mL HGF was selected for use in the following experiments. For the purpose of confirming this result, RT-qPCR was used. As demonstrated in Figure 2E, HGF obviously activated collagen II and fibronectin and inactivated αSMA in TGFβ₁-stimulated HK2 cells. Taken
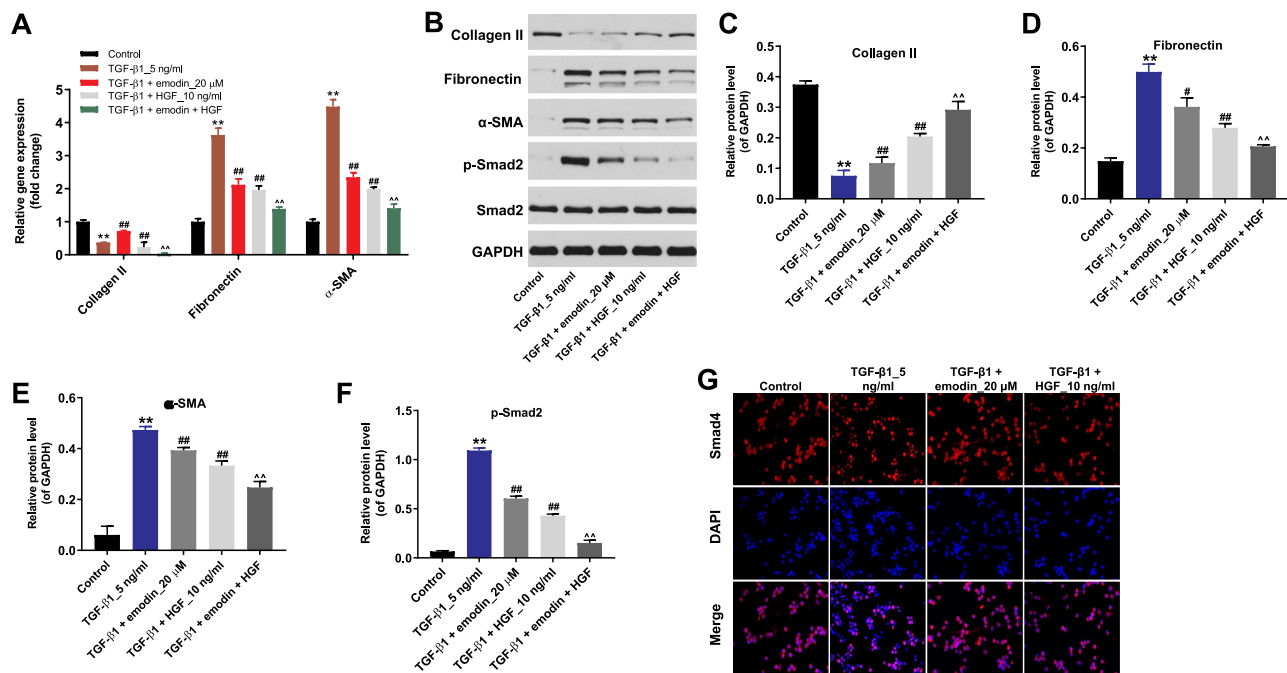
Figure 3 Combination of emodin with HGF suppressed TGFβ₁-induced renal fibrosis via inhibition of the TGFβ₁-signaling pathway. (**A**) After 48 hours' incubation, αSMA, collagen II, and fibronectin expression in HK2 cells was investigated with RT-qPCR. GAPDH was used as a loading control. (**B**) Protein expression of collagen II, fibronectin, αSMA, p-Smad2, and Smad2 in HK2 cells was detected by Western blot. Relative protein expression of (**C**) collagen II, (**D**) fibronectin, (**E**) αSMA, and (**F**) p-Smad2 was quantified by normalizing to GAPDH. (**G**) Smad4 expression in HK2 cells was detected by immunofluorescence staining. **P<0.01 vs control group; #P<0.05 vs TGFβ₁ group; ##P<0.01 vs TGFβ₁ group; ^^P<0.01 vs TGFβ₁ + HGF group.

together, all these data revealed that HGF significantly suppressed the fibrotic effect of TGFβ₁ in HK2 cells.

Combination Treatment Exhibited Significant Suppressive Effect on Renal Fibrosis In Vitro Through Inactivation of TGFβ₁ Pathway

To investigate gene expression in vitro, RT-qPCR was used. As shown in Figure 3A, the expression of collagen II in TGFβ₁-stimulated HK2 cells was notably upregulated by HGF and further increased by emodin. In contrast, HGF notably inhibited the expressions of αSMA and fibronectin in TGFβ₁-treated HK2 cells. As expected, the inhibitory effect of HGF on αSMA and fibronectin was further enhanced in the presence of emodin (Figure 3A). Additionally, protein expression of fibronectin, αSMA, and p-Smad2 in TGFβ₁-treated HK2 cells was significantly decreased by HGF and/or emodin (Figure 3B–F). In contrast, TGFβ₁-induced decrease of collagen II in HK2 cells was notably reversed by HGF and/or emodin. Consistently, the combination of HGF with emodin exhibited better effect on protein expressions compared with either treatment alone (Figure 3B–F). Also, data on Smad4 staining revealed that

TGFβ₁-induced upregulation of Smad4 expression in nuclei of HK2 cells was significantly decreased by HGF and/or emodin (Figure 3G). To sum up, combination treatment exhibited a significant suppressive effect on renal fibrosis in vitro via inactivation of the TGFβ₁ pathway.

Emodin in Combination with HGF Significantly Attenuated Symptoms of UUO-Induced Mouse Renal Fibrosis In Vivo

To investigate the antifibrotic effect of emodin in combination with HGF in vivo, a UUO mice model was established. The results revealed that HGF or emodin notably suppressed renal fibrosis in UUO-treated mice. Moreover, renal fibrosis in UUO-treated mice was further attenuated by combination treatment (Figure 4A). Renal fibrosis symptoms were significantly induced by UUO treatment, which was obviously attenuated by emodin, HGF, or combination treatment (Figure 4B). Fibrosis area was significantly upregulated in UUO-treated mice and significantly downregulated by emodin or HGF alone. Consistently, combination treatment showed more significant antifibrotic effects than emodin- or HGF-alone treatment (Figure 4C). ELISA data demonstrated that HGF and/or emodin notably inhibited levels of Cr and BUN in sera of HFD-treated mice (Figure 4D and E).
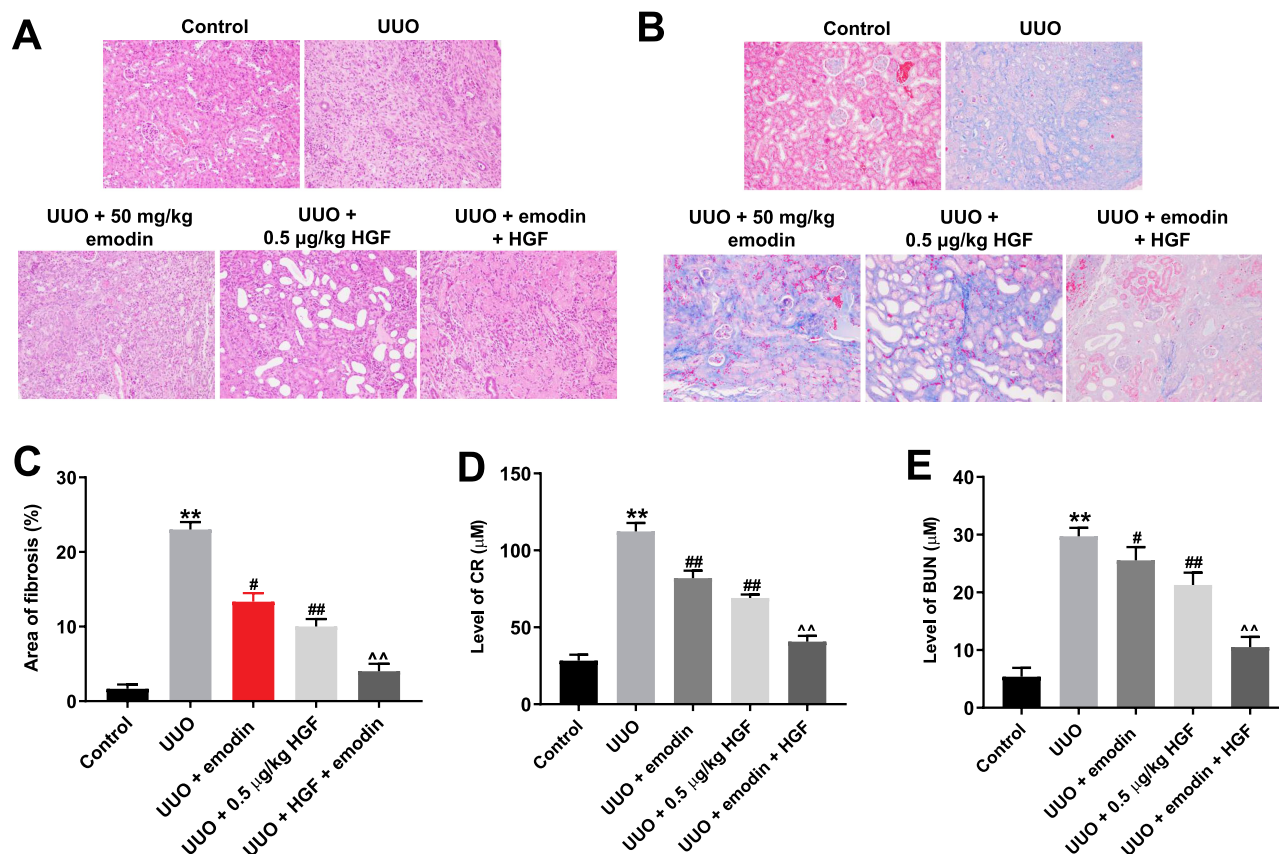
Figure 4 Emodin in combination with HGF notably attenuated UUO-induced renal fibrosis of mice in vivo. (**A**) H&E staining of mouse-kidney tissue in control, UUO, UUO plus 50 mg/kg emodin, UUO plus 0.5 μg/kg HGF, and UUO + emodin + HGF. (**B**) Masson staining of mouse-kidney tissue in control, UUO, UUO plus 50 mg/kg emodin, UUO plus 0.5 μg/kg HGF, and UUO + emodin + HGF group. (**C**) Fibrosis area in mice was quantified. (**D**) Levels of creatinine in sera of mice were detected with ELISA. (**E**) Levels of BUN in sera of mice were detected with ELISA. **$P<0.01$ vs control group; #$P<0.05$ vs UUO group; ##$P<0.01$ vs UUO group; ^^$P<0.01$ vs UUO + 0.5 μg/kg HGF group.

Altogether, combination of emodin with HGF exhibited more significant antirenal fibrosis effect in vivo than emodin- or HGF-alone treatment.

Combination of Emodin with HGF Suppressed Progression of Renal Fibrosis via Inactivation of TGFβ₁ Pathway In Vivo

Finally, to confirm the mechanism by which emodin in combination with HGF relieved renal fibrosis symptoms, collagen II, αSMA, and p-Smad2 expression in mice was detected. As shown in Figure 5A–D, emodin or HGF significantly inactivated αSMA and p-Smad2 in tissue of UUO-treated mice. Moreover, combination treatment further decreased expression of these two protein. In contrast, protein level of collagen II in tissue of UUO-treated mice was greatly upregulated by HGF and/or emodin. Taken together, combination of emodin with HGF attenuated symptoms of renal fibrosis via inactivation of the TGFβ₁ pathway in vivo.

Discussion

In this research, we sought to detect the antifibrotic effect of combination treatment. It has been confirmed that transplantation of HGF-transgenic mesothelial cell sheets show therapeutic effects on renal fibrosis[26] and can be used as an antifibrotic agent.[27] The present study verified that HGF suppressed the progression of renal fibrosis at different concentrations. Emodin has already been confirmed to be able to be used for the treatment of multiple diseases.[22,28] In addition, recent reports have revealed that emodin plays an inhibitory role in neuropathy and hepatopathy.[16,29] Some natural properties showantifibrotic ability.[30,31] Our research was consistent with these studies, further confirming that emodin can be considered an antifibrotic agent. Briefly, our findings strongly confirm that combination treatment shows more significant antifibrotic effects than HGF or emodin alone.

Combination treatment exhibited significant antifibrotic effects via inactivating fibronectin and αSMA and activating collagen II. Previous research has indicated that fibronectin plays a crucial role in fibrosis.[32] Moreover, Notch3
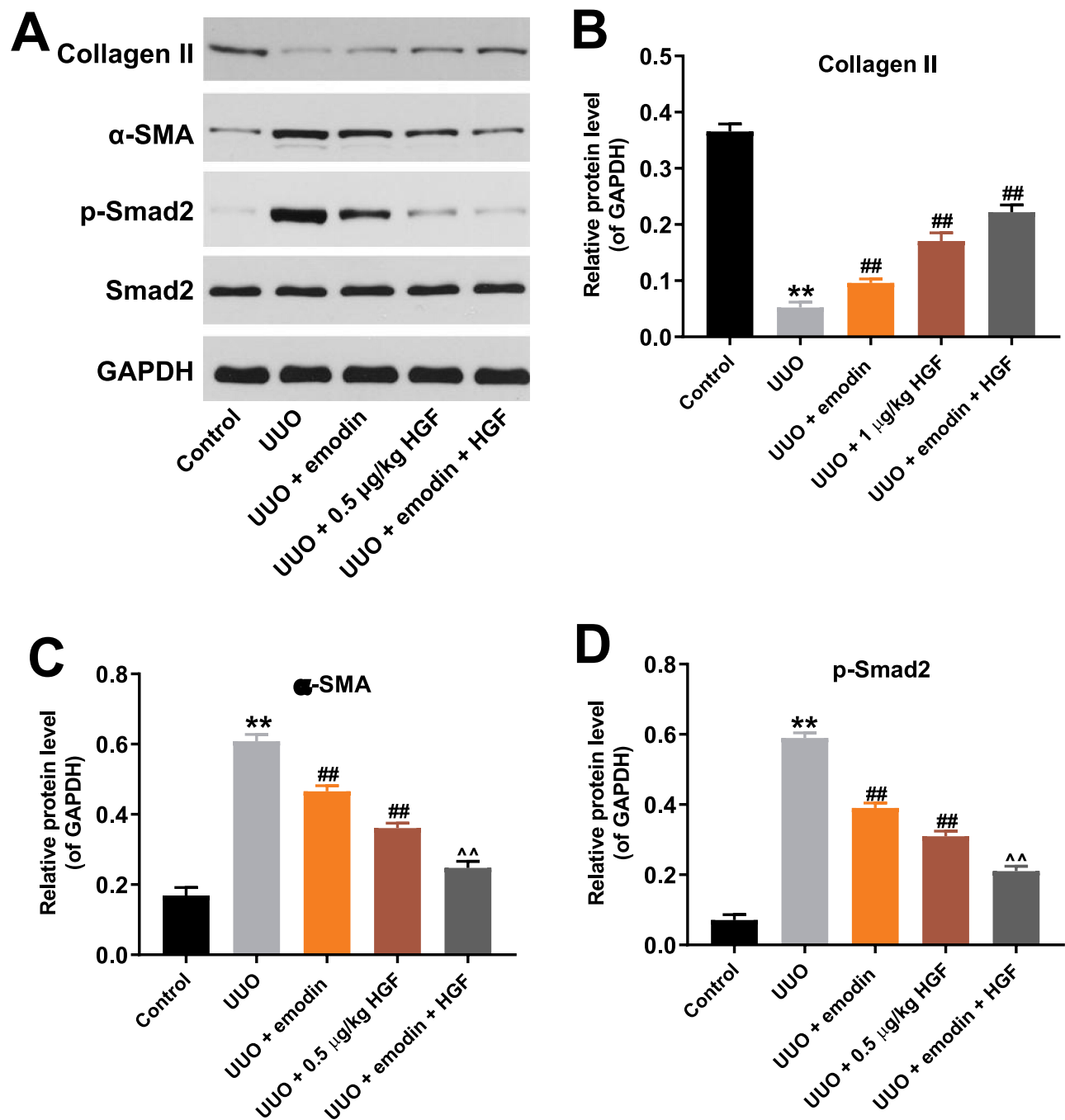
Figure 5 Emodin in combination with HGF inhibited renal fibrosis in vivo by suppression of TGFβ$_1$ pathway. (**A**) Protein expression of collagen II, αSMA, Smad2, and p-Smad2 in kidney tissues of mice was measured by Western blot. Relative protein expression of (**B**) collagen II, (**C**) αSMA, and (**D**) p-Smad2 were quantified by normalizing to GAPDH. **$P<0.01$ vs control group; ##$P<0.01$ vs UUO group; ^^$P<0.01$ vs TGFβ$_1$ + HGF group.

relieves symptom of cardiac fibrosis via downregulation of αSMA.[25] Collagen II has been regarded as being downregulated during the progression of fibrosis.[33] Consistent with these data, emodin in combination with HGF suppressed the development of renal fibrosis via decreasing fibronectin and αSMA and upregulating collagen II in vitro. αSMA plays key roles in epithelial–mesenchymal transition (EMT).[34–36] In this study, the findings indicated that HGF can inactivate αSMA. Emodin significantly enhanced the suppressive effect of HGF on this protein. These data are similar to a recent report, suggesting that HGF and/or emodin exhibit antifibrotic effects via suppression of the EMT process.

TGFβ1 signaling has been confirmed to be involved in fibrosis,[37,38] and is activated persistently in fibrosis.[39,40] Smad4 is at the core of the TGFβ1-signaling pathway.[41] In addition, it has been verified that TGFβ1 can upregulate Smad2, which can affect this signaling pathway, resulting in fibrosis.[42] In the current research, combination treatment further inhibited the expression of p-Smad2 in HK2 cells after TGFβ1 treatment compared with HGF or emodin alone. Based on these data, the mechanism underlying the antifibrotic effects of emodin in combination with HGF was closely correlated with inactivation of TGFβ1 pathways. In recent research, emodin relieved the severity of fibrosis via downregulation of TGFβ1–Smad signaling.[43] Additionally, emodin relieved fibrosis symptoms via inactivation of TGFβ1–Smad signaling.[44] Our study was consistent with these results. Also, our study revealed that αSMA was notably activated in HK2 cells after TGFβ1 treatment. Sisto et al revealed that activation of TGFβ1 signaling can contribute to the upregulation of EMT in Sjögren's syndrome.[45] Consistently, our findings suggested that TGFβ1 signaling upregulated EMT in fibrosis. To sum up, emodin has the potential ability to downregulate fibrosis by inhibiting TGFβ1–Smad signaling. Frankly speaking, our research has paid attention only to the effect of emodin in combination with HGF on the TGFβ1 pathway so far. We will detect the effect of combination treatment on other signaling pathways in future. We have revealed that emodin in combination with HGF shows significantly antifibrotic effects via inactivation of TGFβ1 signaling. Therefore, the combination of emodin with HGF can be used as an effective tool in the treatment of renal fibrosis.

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Disclosure

The authors report no conflicts of interest for this work.

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