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LABORATORY STUDY

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Curcumin mediates repulsive guidance molecule B (RGMb) in the treatment mechanism of renal fibrosis induced by unilateral ureteral obstruction

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ABSTRACT

In this study, we explored the role and mechanism of repulsive guidance molecule B (RGMb, also known as Dragon) in the protective effects of curcumin against renal fibrosis and verified Dragon's effect on renal tubular epithelial cell apoptosis and cell programmability. Unilateral ureteral obstruction (UUO) was surgically induced in rats to establish a model of renal interstitial fibrosis (RIF). The rats were then treated with curcumin. Curcumin prominently decreased the serum creatinine (SCr) and blood urea nitrogen (BUN) levels, and also improved the tubular injury in the UUO-induced rats. Curcumin significantly downregulated the TGF-β1, P-Smad2/3, cleaved caspase-3, cleaved caspase-8 and Dragon levels. Dragon knockdown also markedly reduced the TGF-β1, P-Smad2/3, Smad2/3, cleaved caspase-3, cleaved caspase-8, fibronectin, collagen I, collagen IV, vimentin, and α-SMA expression levels. Conversely, Dragon overexpression caused higher expression levels of these proteins, and curcumin reversed this effect. Furthermore, Dragon knockdown increased the E-cadherin levels, whereas Dragon overexpression decreased these levels. Overexpressing Dragon significantly decreased the cell viability, and curcumin reversed this effect. In conclusion, curcumin acted on Dragon and attenuated RIF in UUO rat models. Curcumin downregulated the TGF-β1/Smad signaling pathway and inhibited Dragon and fibrogenic molecules in both rats and HK-2 cells.

Abbreviations: UUO: unilateral ureteral obstruction; RGMb: repulsive guidance molecule B; TGF-β1: transforming growth factor β1; RIF: renal interstitial fibrosis; EMT: epithelial-mesenchymal transdifferentiation; ECM: extracellular matrix; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; DMSO: dimethyl sulfoxide.

1. Introduction

Renal interstitial fibrosis (RIF) is a common path for all chronic kidney diseases to end-stage renal failure. The main pathological features are interstitial extracellular matrix (ECM) aggregation, inflammatory cell infiltration, and renal tubular epithelial atrophy or shedding. The pathogenesis of RIF is unclear; thus, exploring the mechanisms of RIF has great scientific value and clinical significance [1]. Research has shown that renal tubular epithelial cells actively participate in RIF occurrence and development in various ways. Animal models and pathological specimens from various kidney diseases in humans have shown that areas with heavier interstitial fibrosis have more apoptosis, and the degree of interstitial fibrosis is positively correlated with the amount of apoptosis [2]. Apoptosis or necrosis is an important

pathogenesis of RIF; therefore, in-depth study of renal tubular epithelial death mechanisms is an effective means of clarifying the pathological mechanisms of RIF.

Apoptosis of renal tubular epithelial cells is an important factor in triggering RIF occurrence and progression. Cell death triggers an inflammatory response, inflammatory cell infiltration, cytokine release, increased ECM synthesis and interstitial deposition, leading to renal fibrotic lesions [3]. Experimental studies have shown that inhibiting cell death can downregulate infiltration of renal interstitial inflammatory cells and ECM aggregation, thus slowing RIF. Transforming growth factor-β (TGF-β) is an important molecule that promotes the development of renal fibrosis, and functions through different signaling pathways. For example, TGF-β1 induces the expression of connective tissue

growth factor [4]. Upregulated matrix metalloproteinases in epithelial cells and fibroblasts promote epithelial-mesenchymal transdifferentiation (EMT) and other mechanisms and increase interstitial ECM deposition [5]. Studies have confirmed that TGF-β1 accelerates caspase-3 activation by mediating the MAP kinase and Smad signaling pathways and induces apoptosis of nonimmortalized renal tubular epithelial cells. Various experimental models of renal fibrosis have fully demonstrated the antifibrotic effects of inhibiting TGF-β1 [6–8].

Current research has shown that transdifferentiation of epithelial cells into myofibroblasts is a vital mechanism involved in RIF. EMT is regulated by various cytokines, growth factors and hormones, among which, TGF-β1 is the most important profibrotic factor, mainly through the Smad signaling pathway, to induce expression of its downstream factors to start and regulate the entire EMT process [9].

Curcumin, an active component of turmeric, was first reported to have antibacterial functions in 1949 [10]. Recent studies have demonstrated the traditional role of turmeric (promoting blood circulation and relieving menstrual pain) and revealed new pharmacological effects, including anti-inflammatory effects, antioxidation, liver and kidney protection, and antifibrotic and anticancer effects. These effects may occur by inhibiting the activation and expression of transcription factors such as activator protein-1 and NF-κB, with no obvious toxic or adverse effects.

Repulsive guidance molecule B (RGMb), also known as Dragon, is a member of the RGM family, which consists of RGMa, RGMb, and RGMc. RGM proteins bind to the cell membrane through a glycophosphatidylinositol anchor and have been shown to be coreceptors for bone morphogenetic protein signaling as well as ligands for the neogenin receptor. Previous studies have shown that Dragon is highly expressed in the kidneys and promotes apoptosis of renal tubular epithelial cells subjected to unilateral ureteral obstruction (UUO) [11]. Dragon also plays inhibitory roles in different cancers [12,13].

Experimental UUO in rodents is thought to rapidly mimic human chronic obstructive nephropathy [14]. UUO can cause kidney inflammation and fibrosis, which can in turn cause tubular damage and changes in renal hemodynamics and metabolism. Increasing evidence suggests that curcumin has a protective effect against renal fibrosis, but the mechanism of this effect remains unclear [15,16]. Here, we demonstrated how curcumin mediated Dragon in inhibiting renal fibrosis. We established a UUO-induced rat model of RIF to observe how curcumin affects renal tubular epithelial cell differentiation and the TGF-β1/Smad signaling pathway to explore the mechanism by which curcumin protects against RIF.

2. Methods

2.1. Animals and cells

We used healthy 2–3-month-old male Sprague-Dawley rats weighing 200–250 g obtained from Yunnan University Research Animal Center. The rats were kept in separate cages and fed distilled water and standard rat chow in a pathogen-free environment with a 12-h light/12-h dark cycle. Rats were randomly divided into either the sham ($n = 10$), UUO ($n = 10$) or UUO + curcumin ($n = 10$) groups. The curcumin treatment was started after UUO in rats. After the experiment, laboratory rats were euthanized *via* carbon dioxide (CO_2) asphyxiation. Compressed CO_2 gas was pumped *via* cylinders into the rats' cages, displacing the air in the chamber at a rate of 10–30% per minute. The Ethics Committee of The First People's Hospital of Yunnan Province, The Affiliated Hospital of Kunming University of Science and Technology (Kunming, China) approved all animal experiments (Permit Number: 2018GJ178). Human proximal tubular epithelial (HK-2) cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Hyclone, USA). Curcumin (Sigma, USA) was suspended in 2% dimethyl sulfoxide (DMSO) with 98% saline, at a final concentration >0.1% (v/v) in all experiments. The rats were gavaged with 50 mg/kg or 100 mg/kg curcumin daily, and 10 μM, 20 μM or 30 μM of curcumin were used in the HK-2 cells. Rats in the sham and UUO groups were gavaged with 1% DMSO in saline. In the TGF-β1 induction step, cells were treated with 5 ng/mL TGF-β1 for 2 days before the subsequent experiments [17].

2.2. UUO surgery

UUO surgery was performed by incising through the left flank into the abdomen as per previous studies [14]. After anesthetizing the rats *via* an intraperitoneal injection of sodium pentobarbital, the ureter was identified and tied to the lower pole of the kidney with three separate silk ties. The sham-operated rats underwent a similar procedure, but without ureteral ligation. The rats were euthanized 7 days after UUO surgery.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected from the rats and centrifuged for 15 min at 400 ×g. Serum creatinine (SCr) and blood urea nitrogen (BUN) levels were measured *via* ELISA kit (DAKEWE, China) per the manufacturer's instructions.

2.4. Hematoxylin and eosin (HE) staining

The kidney tissues were fixed and then embedded in paraffin. Then, 2-μm sections of paraffin-embedded kidney tissues were stained with HE commercial kits (Beyotime, China) according to the manufacturer's protocols. To assess renal tubule interstitial injury, the HE-stained sections were semi-quantitatively evaluated as previously described [18]. The renal tubular damage was graded on a scale from 0 to 5 (0, no changes; 1, changes affecting < 10%; 2, changes affecting 11 to 25%; 3, changes affecting 26 to 45%; 4, changes affecting 46 to 75%; and 5, changes affecting ≥76% of the section).

2.5. Cell viability assay

A cell counting kit-8 (CCK-8) was used to detect the viability of HK-2 cells (with or without curcumin treatment). Cells in the logarithmic-growth phase were seeded into 96-well plates at 5×10^4/well and cultured overnight. After rewarming, 10 μL of CCK-8 was added to each cell group, and the cells were incubated for 1 h. The cell absorbance at 450 nm was measured using a microplate reader.

2.6. siRNA interference

HK-2 cells were transfected with non-targeting control siRNA or Dragon siRNA using Lipofectamine 2000 Transfection Reagent (Thermo Scientific). The human Dragon siRNA sequences used were sense: 5′-AUUUAA AGUAUUUACACACUG-3′; antisense: 5′-GUGUGUAAAUA CUUUAAAUUA-3′, and the negative control sequences used were sense: 5′-UUCUCCGAACGUGUCACGUTT-3′; antisense: 5′-ACGUGACACGUUCGGAGAAGT-3′. According to the previous work [19], non-targeting control siRNA and Dragon siRNA sequences were purchased from Ambion Corporation. The concentrations of control siRNA and Dragon siRNA were 60–80 nM. After transfection for approximately 24 h, the HK-2 cells were used in real-time PCR, CCK-8 and western blot assays.

2.7. Dragon cDNA transfection

PcDNA-Dragon plasmids were obtained from GenePharma Company (Shanghai, China). Plasmids were transfected into the HK-2 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After transfection with pcDNA (empty vector) or pcDNA-Dragon, cells were treated with 0 μM, 20 μM or 30 μM curcumin for 3 days.

2.8. RT-qPCR

The rats' kidneys were removed 7 days postsurgery. Cells were cultured in 6-well plates and treated with the indicated concentration of curcumin for 24 h. Total RNA was extracted using TRIzol reagent (Invitrogen, Paisley, UK). The purity and concertation of the extracted RNA were determined using the NanoDrop™ ND-1000. A PrimeScript™ RT reagent kit was used to prepare the cDNA (Takara, Japan). The primers used were F-Dragon-Rat: 5′-GGATGCTATTGAGTCCTGGTG-3′ and R-Dragon-Rat: 5′-CACTGTCTTCTGTTATGTCCC-3′; F-Dragon-human: 5′-GTCAGGAGCAGTAATCTGAG-3′ and R-Dragon-human: 5′-GGCAAAAGTAACTACCTGGAG-3′. RT-qPCR was performed using SYBR Green PCR master mix (Takara, Japan). RT-qPCR amplification was performed in triplicate. RNA expression was calculated *via* the $2^{-\Delta\Delta Ct}$ method [20]. The mRNA expression was normalized against GAPDH to compare the mRNA levels.

2.9. Western blotting

Kidney tissues or HK-2 cells were washed 3 times with phosphate-buffered saline (PBS), then lysed using lysis buffer. Total protein was extracted in radioimmunoprecipitation buffer, separated on polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF), and probed overnight at 4 °C with anti-Dragon, anti-TGF-β1, anti-Smad2/3, anti-cleaved caspase-3, anti-cleaved caspase-8, anti-fibronectin, anti-collagen I, anti-collagen IV, anti-vimentin, anti-E-cadherin or anti-α-smooth muscle actin (α-SMA) antibodies (Abcam, Cambridge, UK). The PVDF membranes were washed 3 times with PBS-Tween-20 (PBST), then treated with the corresponding secondary antibodies. Finally, the membranes were processed using the ECL kit for color reaction.

2.10. Statistical analysis

SPSS version 26.0 and GraphPad Prism 8 were used for data analysis. The results were presented as means ± standard deviations (SD). Student's t-test or one-way analysis of variance (ANOVA) were conducted to calculate the statistical differences. Two-way ANOVA was used when there were two different factors. $p < 0.05$ was considered statistically significant. Image J was used to perform semiquantitative analyses.
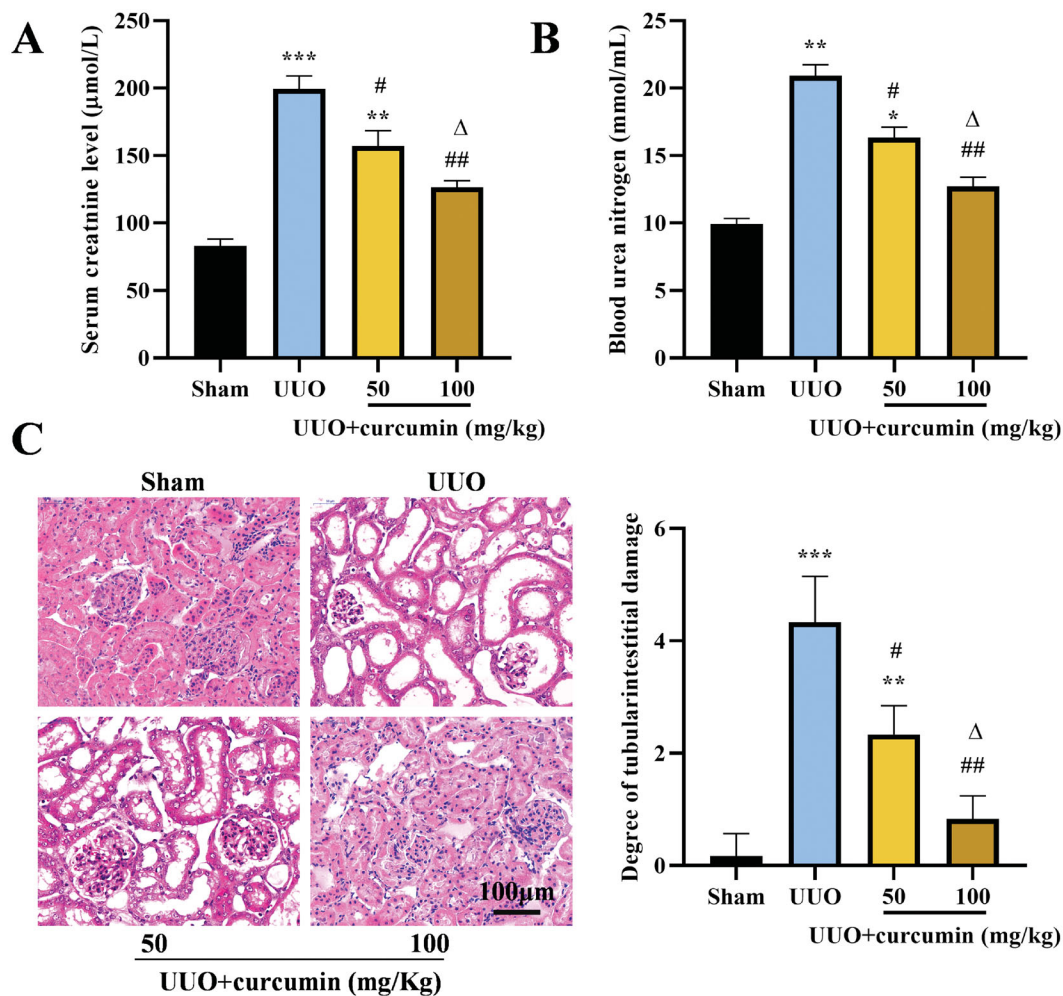
Figure 1. Curcumin improved the renal function of UUO rats. ELISA was used to detect the serum creatinine (A) and blood urea nitrogen (B) levels in the sham, UUO, and UUO + curcumin (50 and 100 mg/kg body weight) groups. (C) Representative micrographs of HE staining of rat kidney tissues and renal injury scores based on HE staining. Scale bar = 100 μm. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. the sham group, $#p < 0.05$, $##$ $p < 0.05$ vs. the UUO group, $\Delta p < 0.05$ vs. the 50 mg/kg curcumin group.

3. Results

3.1. Curcumin ameliorated renal function in UUO rats

To investigate the effect of curcumin on renal function in UUO rats, we measured the SCr and BUN levels (Figure 1). The SCr and BUN levels were markedly increased in the UUO rats compared with those of the sham rats, but these levels were prominently decreased in the UUO rats treated with curcumin. The SCr and BUN levels were significantly lower in the UUO rats treated with 100 mg/kg curcumin than in those treated with 50 mg/kg curcumin ($p < 0.05$). The morphology changes were also examined by HE staining in UUO-induced renal fibrosis with or without the curcumin treatment. As shown in Figure 1(C), the renal pathological changes including tubular necrosis, brush border loss, cast formation and tubular dilation caused by UUO

surgery were improved by curcumin treatment. The renal tubular damage score was markedly lower ($p < 0.001$) in the UUO + curcumin group compared with that in the UUO group, the higher concentration (100 mg/kg) of curcumin possessed a better effect in comparison with the lower concentration (50 mg/kg) of curcumin ($p < 0.05$).

3.2. Curcumin affected TGF-β1 and Smad2/3 phosphorylation in rats

To explore the effect of curcumin on TGF-β1/Smad2/3 signaling, we performed western blot analyses to detect the TGF-β1 and P-Smad2/3 levels in the sham, UUO, and UUO + curcumin (50 and 100 mg/kg body weight) groups ($n = 10$ rats per group). TGF-β1 and P-Smad2/3 levels were highly elevated in the UUO group compared with those of the sham group (Figure 2(A)).
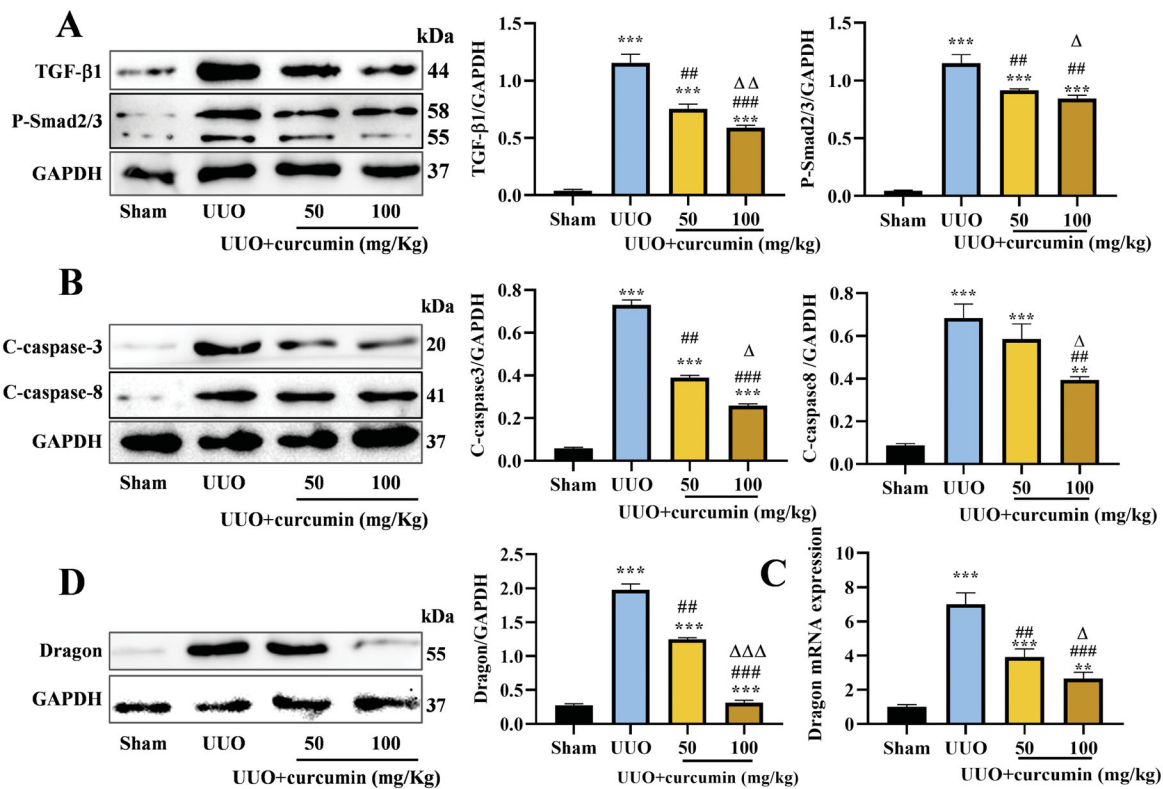
Figure 2. Curcumin inhibited TGF-β1, P-Smad2/3 and apoptosis and decreased Dragon expression *in vivo*. (A) Western blot analysis of TGF-β1 and P-Smad2/3 expression in the sham, UUO, and UUO + curcumin (50 and 100 mg/kg body weight) groups. TGF-β1 and P-Smad2/3 expressions were quantified *via* densitometric analysis. (B) Western blot analysis of cleaved caspase-3 (C-caspase-3) and cleaved caspase-8 (C-caspase-8) expression in the sham, UUO, and UUO + curcumin (50 and 100 mg/kg body weight) groups. Cleaved caspase-3 and cleaved caspase-8 expressions were quantified *via* densitometric analysis. RT-qPCR (C) and western blot analysis (D) of Dragon (repulsive guidance molecule member B) expression in the sham, UUO, and UUO + curcumin (50 and 100 mg/kg body weight) groups. GAPDH was used as the loading control for the western blots. $n = 5$–6. $**p < 0.01$, $***p < 0.001$ *vs.* the sham group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* the UUO group, $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$ *vs.* the 50 mg/kg curcumin group.

Curcumin effectively reduced the TGF-β1 and P-Smad2/3 levels compared with those in the UUO group ($p < 0.001$). The effect of curcumin on apoptosis was also detected by western blotting analysis.

3.3. Curcumin inhibited apoptosis and decreased Dragon expression in rats

Cleaved caspase-3 and cleaved caspase-8 levels were increased in the UUO rats and reduced in the curcumin-treated rats ($p < 0.01$; Figure 2(B)). We also assessed the effect of curcumin on Dragon expression (Figure 2(C,D)). The Dragon mRNA and protein levels were prominently promoted compared with those of the sham rats; however, these levels were highly attenuated in the curcumin-treated group ($p < 0.001$).

3.4. Curcumin treatment downregulated Dragon in fibroblasts activated by TGF-β1 in HK-2 cells

To determine the effects of 10 μM, 20 μM, and 30 μM curcumin on Dragon in RIF, HK-2 cells were cultured

and activated with TGF-β1 (5 ng/mL). RT-qPCR and western blot results showed that the Dragon mRNA and protein expression levels were significantly reduced ($p < 0.001$) in the curcumin-treated cells compared with those of the HK-2 cells without curcumin. Cells treated with 30 μM curcumin had less Dragon expression than did cells treated with 10 μM or 20 μM curcumin at both the mRNA and protein levels (Figure 3).

3.5. Knockdown of Dragon decreased fibrosis and fibroblast proliferation, TGF-β1, P-Smad2/3, and apoptosis induced by TGF-β1 in HK-2 cells

To determine whether inhibiting Dragon would affect the expressions of fibrotic molecules in kidney tissues, HK-2 cells were transfected with negative control siRNA (si-NC) or Dragon siRNA (si-Dragon), and the Dragon mRNA expression levels were assayed. Dragon mRNA expression levels were markedly reduced compared with those the si-NC group and significantly increased with TGF-β1 compared with those of the corresponding
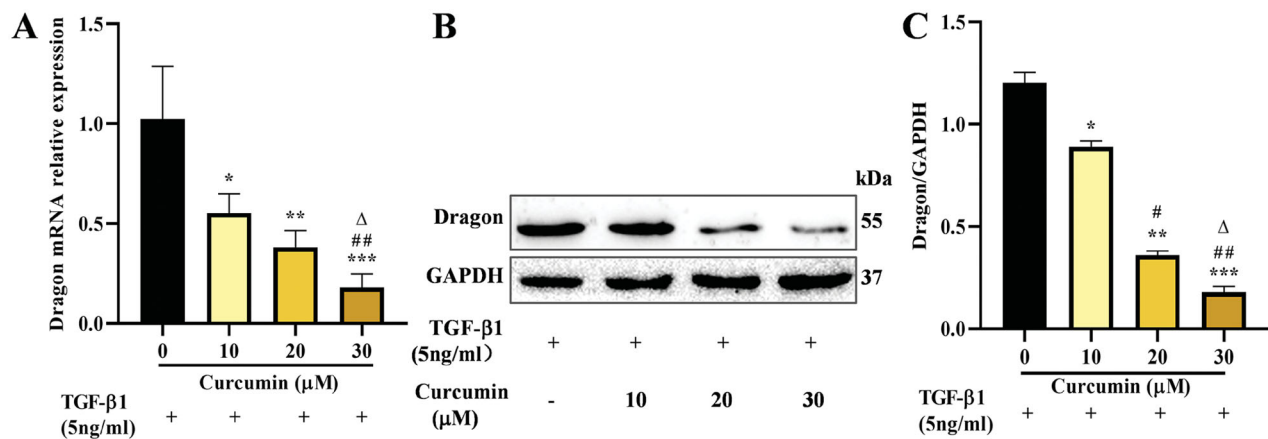
Figure 3. Curcumin downregulated Dragon in TGF-β1-activated fibroblasts in HK-2 cells. (A) RT-qPCR analysis of Dragon expression in the TGF-β1-alone and TGF-β1 + curcumin groups. (B) Western blot analysis of Dragon expression in TGF-β1-alone and TGF-β1 + curcumin groups. (C) Dragon expression was quantified *via* densitometric analysis. GAPDH was used as the loading control for western blots. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ *vs.* the TGF-β1 group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* the 10-μM curcumin group, $^{\Delta}p < 0.05$, *vs.* the 20-μM curcumin group.

si-NC or si-Dragon groups without TGF-β1. Induction of TGF-β1 decreased the Dragon mRNA expression levels in the si-Dragon group (Figure 4(A)). The protein expression levels of key fibrogenic molecules involved in RIF progression, including fibronectin, collagen I, collagen IV, vimentin, E-cadherin and α-SMA, were detected. Fibronectin, collagen I, collagen IV, vimentin, E-cadherin and α-SMA expression levels did not significantly differ between the si-NC and si-Dragon groups without TGF-β1 induction. Fibronectin, collagen I, collagen IV vimentin, α-SMA and TGF-β1 expression levels were highly reduced in the si-Dragon groups compared with those of the si-NC groups in TGF-β1-induced HK-2 cells. E-cadherin expression was significantly increased in the si-Dragon groups compared with those of the si-NC groups in TGF-1-induced HK-2 cells (Figure 4(B,C)). TGF-β1 induction significantly upregulated the main proteins involved in TGF-β1/Smad2/3 signaling in the si-NC and si-Dragon groups. TGF-β1 expression and the relative protein expressions of P-Smad2/3/Smad2/3 were obviously reduced in the si-Dragon group compared with those of the si-NC group. We also detected the effects of Dragon on HK-2 cell proliferation *via* CCK-8 assay. TGF-β1-induced HK-2 cells in the si-Dragon group exhibited significantly lower cell viability than did cells in the other groups (Figure 4(D)). Apoptosis was examined by detecting the protein expression levels of initiator caspase cleaved caspase-8 and executioner caspase cleaved caspase-3. The cleaved caspase-3 and cleaved caspase-8 expression levels were markedly elevated in the TGF-β1-treated groups compared with those of the groups without TGF-β1 treatment as well as in the si-Dragon groups compared with those of the si-NC groups (Figure 4(F)).

3.6. Curcumin reversed fibrosis, fibroblast proliferation, TGF-β1 and P-Smad2/3 expressions and apoptosis induced by Dragon overexpression

We next examined whether Dragon mediated curcumin's function in RIF. PcDNA-Dragon was constructed to overexpress Dragon, and the empty pcDNA vector served as the control. Fibronectin, collagen I, collagen IV, vimentin, E-cadherin, α-SMA, TGF-β1, cleaved caspase-3 and cleaved caspase-8 expressions as well as the relative P-Smad2/3/Smad2/3 expression did not obviously change as the curcumin concentration increased in the pcDNA groups (Figure 5(A–F)). Conversely, these expressions were all significantly reduced as the curcumin concentration increased ($p < 0.05$). E-cadherin expression increased as the curcumin dose increased ($p < 0.05$). HK-2 cell proliferation remained at a relatively constant level in the pcDNA groups but was significantly reduced in the pcDNA-Dragon groups as the curcumin concentration increased (Figure 5(G)).

4. Discussion

Renal fibrosis is the usual pathway by which nearly all kidney diseases develop into end-stage renal failure; thus, preventing and even reversing kidney fibrosis early is of great significance in preventing and treating end-stage renal failure [21].

Curcumin has shown antifibrotic effects and has received much attention in treating RIF and other kidney diseases [22]. As per previous studies, we used 100 mg/kg of curcumin in rats to study renal fibrosis [23]. Even at 50 mg/kg curcumin, the TGF-β1 and P-Smad2/3 protein levels were effectively reduced compared with those of the UUO group. Curcumin also
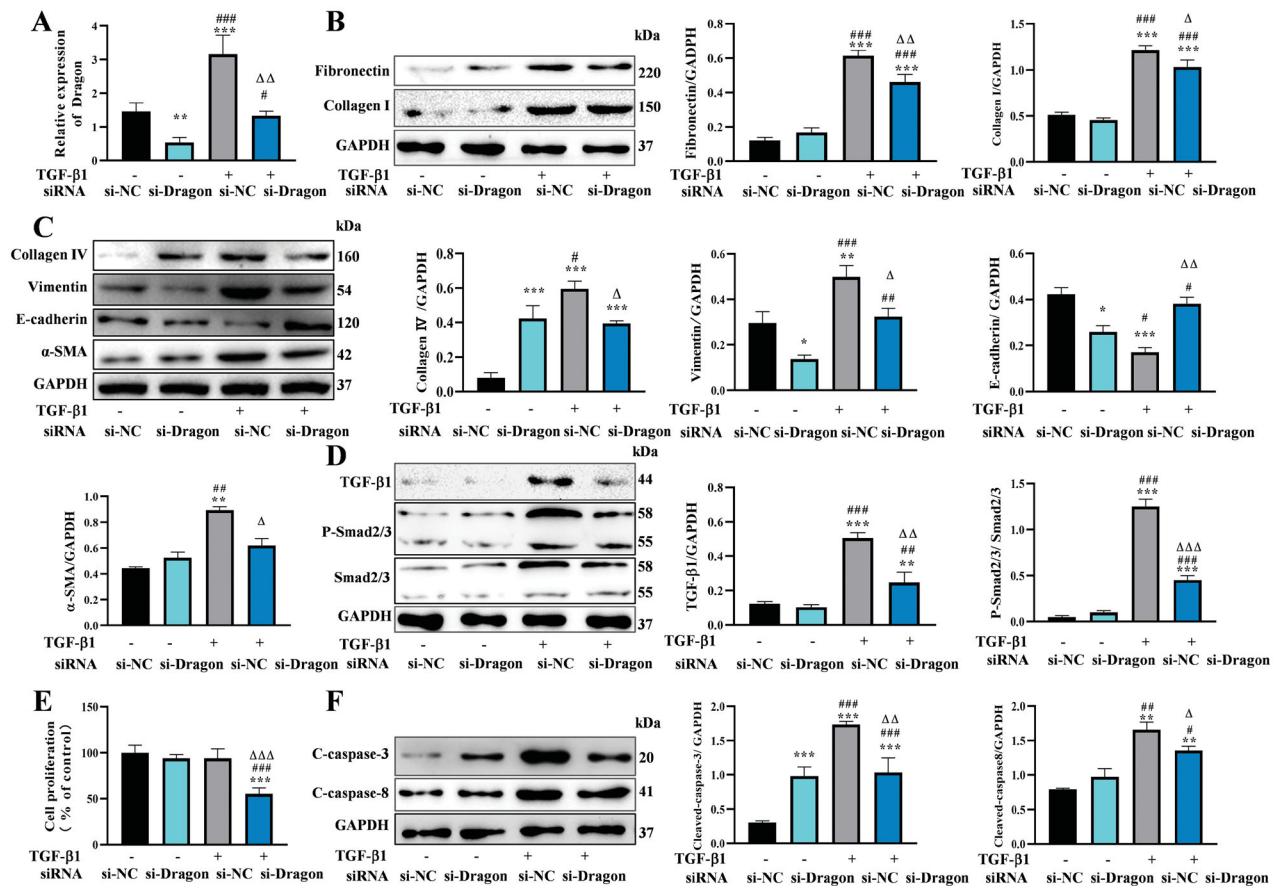
Figure 4. Knockdown of Dragon decreased fibrosis and fibroblast proliferation, TGF-β1, P-Smad2/3, Smad2/3 and apoptosis induced by TGF-β1 in HK-2 cells. Cells were transferred with non-targeting control siRNA (si-NC) or Dragon si-RNA (si-Dragon) (A) RT-qPCR of Dragon expression in the si-NC-alone, si-Dragon-alone, si-NC + TGF-β1, and si-Dragon + TGF-β1 groups. Western blot analysis of (B) fibronectin, collagen I, (C) collagen IV, vimentin, E-cadherin and α-SMA expression. Fibronectin, collagen I, collagen IV, vimentin, E-cadherin and α-SMA expressions were quantified *via* densitometric analysis. (D) Western blot analysis of TGF-β1and P-Smad2/3 expression. TGF-β1 and relative P-Smad2/3/Smad2/3 expressions were quantified *via* densitometric analysis. (E) CCK-8 assay of cell proliferation. (F) Western blot analysis of cleaved caspase-3 and cleaved caspase-8 expression in those four groups. Cleaved caspase-3 and cleaved caspase-8 were quantified *via* densitometric analysis. GAPDH was used as the loading control for the western blots. Each value represents the mean ± SD; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs* si-NC alone, ##$p < 0.01$, ###$p < 0.001$ *vs.* si-Dragon alone, Δ$p < 0.05$, ΔΔ$p < 0.01$, ΔΔΔ$p < 0.001$ *vs.* si-NC with TGF-β1.

induced antiapoptotic effects by significantly reducing the protein levels of cleaved caspase-3 and cleaved caspase-8. Additionally, Dragon and curcumin were negatively correlated in TGF-β1-triggered cells.

We investigated TGF-β1/Smad signaling because this signaling is reported to be a major pathway in the development of renal fibrosis [24,25]. TGF-β has been found to be upregulated in animal models and humans with chronic kidney diseases [26,27]. TGF-β signals can trigger phosphorylation of Smad2 and Smad3. Subsequently, phosphorylated Smad2/3 translocates into nuclei and is involved in controlling TGF-β-responsive gene transcription [28].

TGF-β1 and Smad2/3 phosphorylation levels were markedly increased in the UUO rat model. This was reversed dose-dependently after curcumin treatment. Curcumin treatment also significantly reduced the

elevated TGF-β1 and P-Smad2/3 expressions stimulated by TGF-β1 in HK-2 cells. TGF-β1 promoted renal fibrosis but induced no significant differences in cell proliferation in our experiments.

Recent studies have shown that Dragon promotes renal tubular epithelial cell apoptosis and exacerbates kidney damage. Knocking out Dragon in heterozygous mice increased the expression of E-cadherin, a marker molecule of renal tubular epithelial cell integrity [29]. *In vitro* cell culture experiments showed that Dragon overexpression promoted epithelial cell apoptosis [30]. In our study, knocking down Dragon decreased apoptosis of cells expressing cleaved caspase-3 and cleaved caspase-8; overexpressing Dragon increased this apoptosis.

Excessive deposition of ECM components, such as fibronectin and types I and III collagen, contributes to renal fibrosis development [23]. Fibronectin, collagen I,
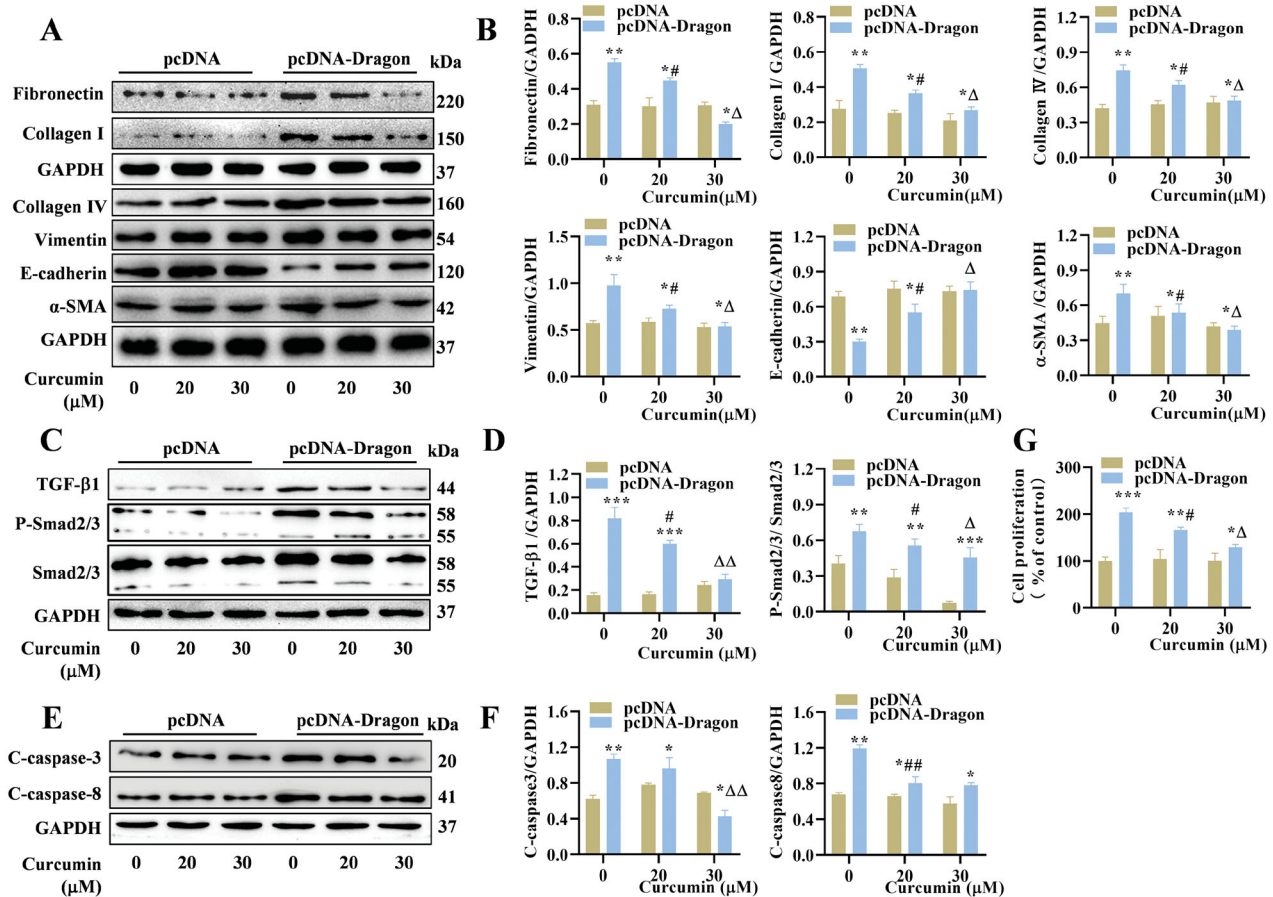
Figure 5. Curcumin reversed fibrosis, fibroblast proliferation, TGF-β1 and P-Smad2/3 expression and apoptosis induced by Dragon overexpression. (A) Western blot analysis of fibronectin, collagen I, collagen IV, vimentin, E-cadherin and α-SMA expressions in the groups with or without Dragon at different concentrations of curcumin. (B) Fibronectin, collagen I, collagen IV, vimentin, E-cadherin and α-SMA expressions were quantified *via* densitometric analysis. (C) Western blot analysis of TGF-β1, Smad2/3 and P-Smad2/3. (D) TGF-β1 and relative P-Smad2/3/Smad2/3 expressions were quantified *via* densitometric analysis. (E) Western blot analysis of cleaved caspase-3 and cleaved caspase-8 expressions in those six groups. (F) Cleaved caspase-3 and cleaved caspase-8 expression were quantified *via* densitometric analysis. (G) CCK-8 assay of fibroblast proliferation. GAPDH was used as the loading control for the western blots. Each value represents the mean ± SD; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ *vs.* pcDNA group at the same concentration of curcumin; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* pcDNA-Dragon + curcumin (0 μM); $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$, *vs.* pcDNA-Dragon + curcumin (20 μM).

collagen IV, vimentin, and α-SMA expression levels were stimulated by TGF-β1 and reduced by knocking down Dragon; overexpressing Dragon reversed these effects. E-cadherin expression was inhibited by TGF-1 and increased by knocking down Dragon; overexpressing Dragon reversed these effects. Several drugs have been shown to attenuate renal fibrosis by inhibiting the TGF-β1/Smad signaling pathway [31,32].

In conclusion, curcumin acted on Dragon and attenuated RIF in UUO rat models. Curcumin downregulated the TGF-β1/Smad signaling pathway and inhibited Dragon and fibrogenic molecules in both rats and HK-2 cells. Our results suggest that the TGF-β1/Smad2/3 signaling pathway is involved in the regulation of Dragon by curcumin. Curcumin plays a protective role against renal fibrosis *via* Dragon affecting the TGF-β1/Smad2/3 signaling pathway. Our study may help develop new treatments for patients with renal fibrosis.

Ethical approval

The Ethics Committee of The First People's Hospital of Yunnan Province, The Affiliated Hospital of Kunming University of Science and Technology (Kunming, China) approved this study. Consent to participate was not applicable.

Disclosure statement

Data availability statement

All data generated or analyzed during this study are included in this manuscript.

Authors' contributions

LYX made substantial contributions to conceiving and designing the study. FC, YX and QL were responsible for data acquisition and analysis. YX, QL and WZ were responsible for data interpretation. YX and WZ were responsible for data visualization. FC and YX drafted the manuscript. LYX revised the manuscript critically for important intellectual content. All authors have read and agree with the final manuscript.

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