Tauroursodeoxycholic acid inhibits TGF-β1-induced renal fibrosis markers in cultured renal mesangial cells by regulating endoplasmic reticulum stress

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Abstract. Chronic kidney disease (CKD) has a worldwide prevalence of higher than 10% with an increasing mortality rate. As it involves the deterioration of renal function, it represents a serious risk to human health and, if left untreated, significantly lowers the quality of the patient's life. CKD is characterized by renal fibrosis. Studies have shown that transforming growth factor β1 (TGF-β1), a key driving factor of renal fibrosis, is closely related to the activation of renal fibrosis pathways such as endoplasmic reticulum stress (ERS). Tauroursodeoxycholic acid (TUDCA), an endogenous bile acid derivative, can effectively inhibit endogenous ERS. Here, we explored the effects and actions of TUDCA on renal fibrosis by establishing a renal mesangial cell (RMC) model. The RMC was stimulated with TGF-β1, and PCR and western blotting were used to detect the expression of ERS-related chaperone proteins and fibrotic indicators. The expression of glucose-regulated protein 78 (GRP78) was silenced in RMC cells to investigate the role of GRP78 in renal fibrosis. Finally, PCR and western blotting were used to detect the effects of TUDCA on the expression of GRP78, C/EBP homologous protein (CHOP), α-smooth muscle actin (α-SMA), and fibronectin (FN) in the TGF-β1-stimulated RMCs. The results showed that TUDCA significantly downregulated TGF-β1-induced levels of GRP78, CHOP, α-SMA and FN in RMCs. In addition, downregulation of GRP78 inhibited the expression of FN and α-SMA in the RMCs. In conclusion, downregulation of GRP78 and CHOP expression is one of the mechanisms by which TUDCA inhibits TGF-β1-induced renal mesangial cell fibrosis.

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Abbreviations: CKD, chronic kidney disease; TGF-β1, transforming growth factor β1; ERS, endoplasmic reticulum stress; TUDCA, tauroursodeoxycholic acid; RMCs, renal mesangial cells; GRP78, glucose-regulated protein 78; CHOP, C/EBP homologous protein; α-SMA, α-smooth muscle actin; FN, fibronectin; ECM, extracellular matrix; ER, endoplasmic reticulum; siRNA, small interfering RNA; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; COL I, collagen type I; EMT, epithelial-mesenchymal transition; MM, mesangial matrix; TIMPs, tissue inhibitors of metalloproteinases

Key words: chronic kidney disease, renal fibrosis, transforming growth factor β1, tauroursodeoxycholic acid, endoplasmic reticulum stress

Introduction

Chronic kidney disease (CKD), with a global incidence of higher than 10%, is a serious threat to human health (1,2). Moreover, the incidence of CKD is increasing as populations age and diseases such as diabetes, hypertension, and obesity have become more prevalent. Apart from health issues, CKD causes significant economic burdens to both the family and society. CKD is characterized by renal fibrosis, specifically, the deposition of excess extracellular matrix (ECM) in the glomerulus and interstitial area (3). In the absence of effective treatment, renal fibrosis eventually progresses to end-stage renal disease in most CKD patients (4).

The endoplasmic reticulum (ER) is responsible for protein processing and calcium storage. When overwhelmed, the ER accumulates misfolded or unfolded proteins and the calcium balance is disrupted, a condition known as endoplasmic reticulum stress (ERS) (5). Various factors such as hypoxia, drugs, oxidative stress, and abnormal protein glycosylation can lead to the accumulation of unfolded or misfolded proteins, ultimately triggering ERS (6). Recent studies have shown that ERS plays an active role in the development of renal fibrosis in CKD; however, the specific molecular mechanism is unclear (7,8). Transforming growth factor β1 (TGF-β1), a key player in ERS, can drive renal fibrosis (9,10). Notably, in the early stage of ERS, an ERS-related chaperone protein, glucose-regulated protein 78 (GRP78) is upregulated and binds unfolded or misfolded proteins inducing an unfolded protein response. This leads to the upregulation of C/EBP homologous protein (CHOP) that induces cell apoptosis (11). Therefore, inhibition of TGF-β1 signaling, expression of the

ERS molecular chaperone protein, and/or directly blocking ERS may be effective strategies for delaying renal fibrosis.

Tauroursodeoxycholic acid (TUDCA), an endogenous bile acid derivative, is used as a liver protectant in cholestatic liver disease (12). It is also a potent inhibitor of the apoptosis pathway (13,14). Research has shown that advanced glycation end products (AGEs) can induce ERS, while TUDCA can inhibit AGE-induced ERS and, in turn, apoptosis in a dose-dependent manner (15).

In the present study, we used small interfering RNA (siRNA) to examine the effect of GRP78 on TGF-β1-induced renal fibrosis. Furthermore, we explored the mechanism of TUDCA in regulating ERS and affecting the occurrence and development of renal fibrosis in CKD patients. Our results suggest that TUDCA can be a potential therapy for renal fibrosis in CKD patients.

Materials and methods

Cell culture. Rat renal mesangial cells (RMCs) were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc.) with or without 5% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C, 95% O_2 and 5% CO_2 in a humidified atmosphere. RMCs were passaged twice a week. For the study, RMCs were treated with recombinant human TGF-β1, and a control group was also set up.

Cell transfection of siRNA. RMCs were seeded in 6-well plates and cultured for 24 h. Three siRNAs targeting rat *GRP78* and a negative control siRNA were purchased from Jiman Biotechnology Co. Transient transfections were performed using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. After 48 h of transfection, GRP78 expression was detected by western blotting and real-time fluorescent quantitative PCR.

Real-time PCR. Cellular RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. cDNA was synthesized using the PrimeScript™ 1st Strand cDNA Synthesis Kit (D6110A; Takara Bio, Inc.) following the manufacturer's instructions. The gene fragments were then amplified and quantitated by real-time PCR using the following conditions: 1 cycle of 95˚C for 10 min; 95˚C for 10 sec; 60˚C for 15 sec; 72˚C for 20 sec (40 cycles), and 72˚C for 10 min. The starting template was quantitated using the CT value estimated by the real-time PCR recorder. *GAPDH* was used as the internal control. The specific primer sequences are listed in Table SI (also available at URL: https://figshare.com/s/12a0cdf45471f06dc16f; DOI: 10.6084/m9.figshare.16744189).

Western blotting. Cells were harvested, lysed, and the total protein was extracted using a protein extraction kit (P0033, Beyotime Biotechnology), and further quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.) according to the user guide. Protein samples (15 μg) were separated on 10% SDS-PAGE gels and transferred to PVDF membranes followed by blocking in TBST containing 5% skimmed milk for 2 h at room temperature. Primary antibodies were diluted as described below to working concentrations in 1X TBST with 1% skimmed milk and incubated overnight at 4˚C. After three washes with TBST, the membranes were incubated with the appropriate secondary antibodies for 2 h at room temperature with gentle rocking. Finally, the protein bands were visualized with ECL solution for 1 min, photosensitized, and finally analyzed using BanScan software 5.0 (ProZyme; Agilent Technologies, Inc.). The protein levels were normalized to levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The primary antibodies used were as follows: rabbit anti-GRP78 polyclonal antibody (#3183, Cell Signaling Technology, Inc.) diluted at 1:1,000; rabbit anti-α-SMA polyclonal antibody (ab5694, Abcam) diluted at 1:1,000; rabbit anti-collagen type I (COL I) monoclonal antibody (ab138492, Abcam) diluted at 1:500; mouse anti-fibronectin monoclonal antibody (ab6328, Abcam) diluted at 1:1,000; mouse anti-CHOP monoclonal antibody (2895, Cell Signaling Technology, Inc.) diluted at 1:1,000; mouse anti-GAPDH monoclonal antibody (sc-365062, Santa Cruz Biotechnology, Inc.) diluted at 1:400. The secondary antibodies used were horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG H&L (ab205718, Abcam) and HRP-conjugated rabbit anti-mouse IgG (H+L) (ab6728; Abcam) both diluted at 1:2,000.

Immunofluorescence. RMCs, cultured on chamber slides, were fixed with 4% paraformaldehyde and permeabilized with PBS containing 0.1% Triton X-100. The cells were incubated with the respective primary antibodies described above at the following dilutions: mouse anti-fibronectin monoclonal antibody, 1:200; rabbit anti-collagen type I, 1:100; rabbit anti-α-SMA polyclonal antibody, 1:100. Primary antibodies were incubated at 4˚C overnight, followed by incubation with the corresponding secondary antibodies goat anti-rabbit IgG-H&L conjugated to Alexa Fluor® 488 (ab150077, Abcam) diluted at a 1:200 ratio and horse anti-mouse IgG antibody (H+L) conjugated with DyLight® 488 (DI-2488-1.5, Vector Laboratories) diluted at a 1:200 ratio for 2 h at 37˚C. The cell nuclei were counterstained with DAPI for 1 h. The slides were examined under a fluorescence microscope and analyzed with Image J software v1.8.0 (National Institutes of Health).

Statistical analysis. GraphPad Prism 7.0 software (GraphPad Software, Inc.) was used for statistical analysis. Values are expressed as mean ± variance. The t-test was used for the statistical calculation of real-time quantitative PCR data. Data were analyzed using the Wilcoxon rank-sum test for paired comparisons, and the one-way ANOVA test followed by Bonferroni or Fisher LSD post hoc tests for multiple comparisons. P-value <0.05 denotes statistical significance.

Results

TGF-β upregulates ERS-related chaperone proteins and fibrotic factors. Renal fibrosis mainly involves renal mesangial cells. Here, we examined the effect of TGF-β1 on the expression of ERS-related and fibrotic factors at the cellular level. For this, TGF-β1 was used in a concentration- (1, 5 and

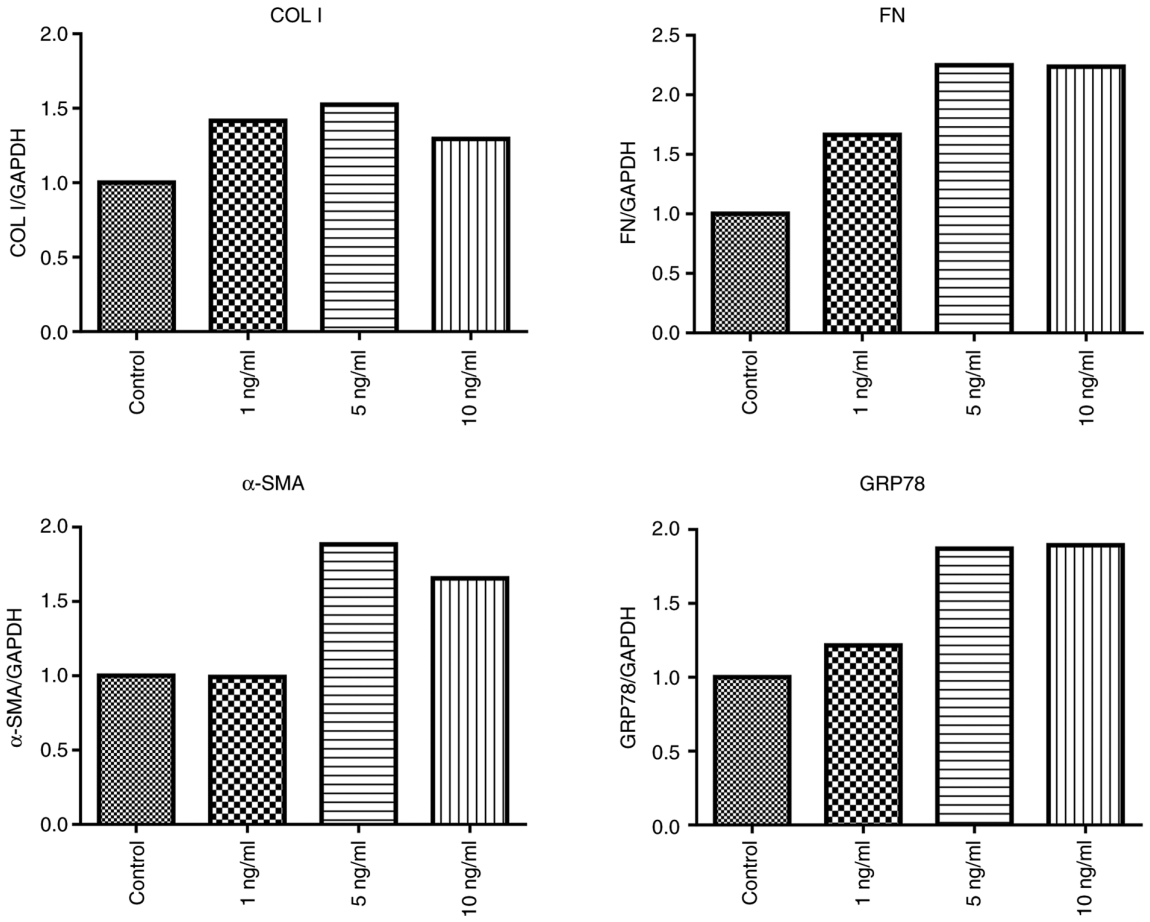

Figure 1. Effects of TGF-β1 on the expression of COL I, FN, α-SMA and GRP78 in RMCs. RMCs were stimulated with TGF-β1 (1, 5 and 10 ng/ml), and gene expression of *COL I*, *FN*, *α-SMA* and *GRP78* was assessed after 24 h by RT-PCR. TGF-β1, transforming growth factor β1; *COL I*, collagen type I; *α-SMA*, α-smooth muscle actin; *FN*, fibronectin; *GRP78*, glucose-regulated protein 78; RMCs, rat renal mesangial cells.

10 ng/ml) and time-gradient (6 and 24 h) manner. We found that after 24 h of treatment, TGF-β1 (5 and 10 ng/ml) stimulated the expression of GRP78 and the pro-fibrotic factors COL I, FN and α-SMA were markedly increased than in the control group (Fig. 1). Thus, we selected 5 ng/ml TGF-β1 for subsequent experiments.

Next, RMCs were stimulated with 5 ng/ml TGF-β1 for 6, 12 and 24 h. We found that the cellular expression of GRP78, the expression of transcription factor CHOP and the expression of fibrosis marker COL I were significantly increased after 24 h compared to the control group (Fig. 2A). Meanwhile, TGF-β1 stimulation also observably increased the expression of the fibrosis marker FN (Fig. 2A), although no significant statistical significance was observed. Furthermore, the protein levels of CHOP and GRP78 were also consistent with the PCR results, while the changes in COL I and FN protein expression differed from their mRNA expression (Fig. 2B). In summary, these observations indicate that TGF-β1 treatment for 24 h significantly increased the expression of ERS-related proteins and fibrotic factors in RMCs, corresponding to the features of renal fibrosis.

Downregulation of GRP78 expression inhibits renal fibrosis. To examine whether ERS is directly related to renal fibrosis, we transiently transfected siRNA-*GRP78* to downregulate the *GRP78* gene in RMCs. Compared with the control group, the three siRNA sequences targeting *GRP78* (siR-1, siR-2 and siR-3) markedly reduced the expression of *GRP78* (51-67%) (Fig. 3A). Western blotting further confirmed that the protein levels of GRP78 were also reduced (Fig. 3B). For subsequent experiments, we selected an siRNA sequence (siR-1) that produced the maximum effect and transiently transfected RMC cells with or without TGF-β1 stimulation. Compared with the control group, transfection of siRNA-*GRP78* (*GRP78*siRNA^{+}) markedly reduced the protein levels of GRP78 in RMCs, while also reducing the levels of the fibrosis markers FN and α-SMA. However, compared with the TGF-β stimulation group, transfection of siRNA-*GRP78* did not significantly downregulate the TGF-β1-induced protein levels of FN and α-SMA (Fig. 3C). These results were verified by immunofluorescence (Fig. 3D). This shows that the downregulation of GRP78 can inhibit fibrosis in RMCs.

TUDCA inhibits the pro-fibrotic effect of TGF-β1. A serum-free medium can be used to eliminate the influence of pro-fibrotic components in the serum. Firstly, we examined the effect of TUDCA on ERS-related proteins in the presence or absence of serum-containing media. The western blotting results showed markedly differences between the two groups (Fig. 4). In the serum-free group, TUDCA markedly inhibited the expression of GRP78 and FN proteins in the RMCs

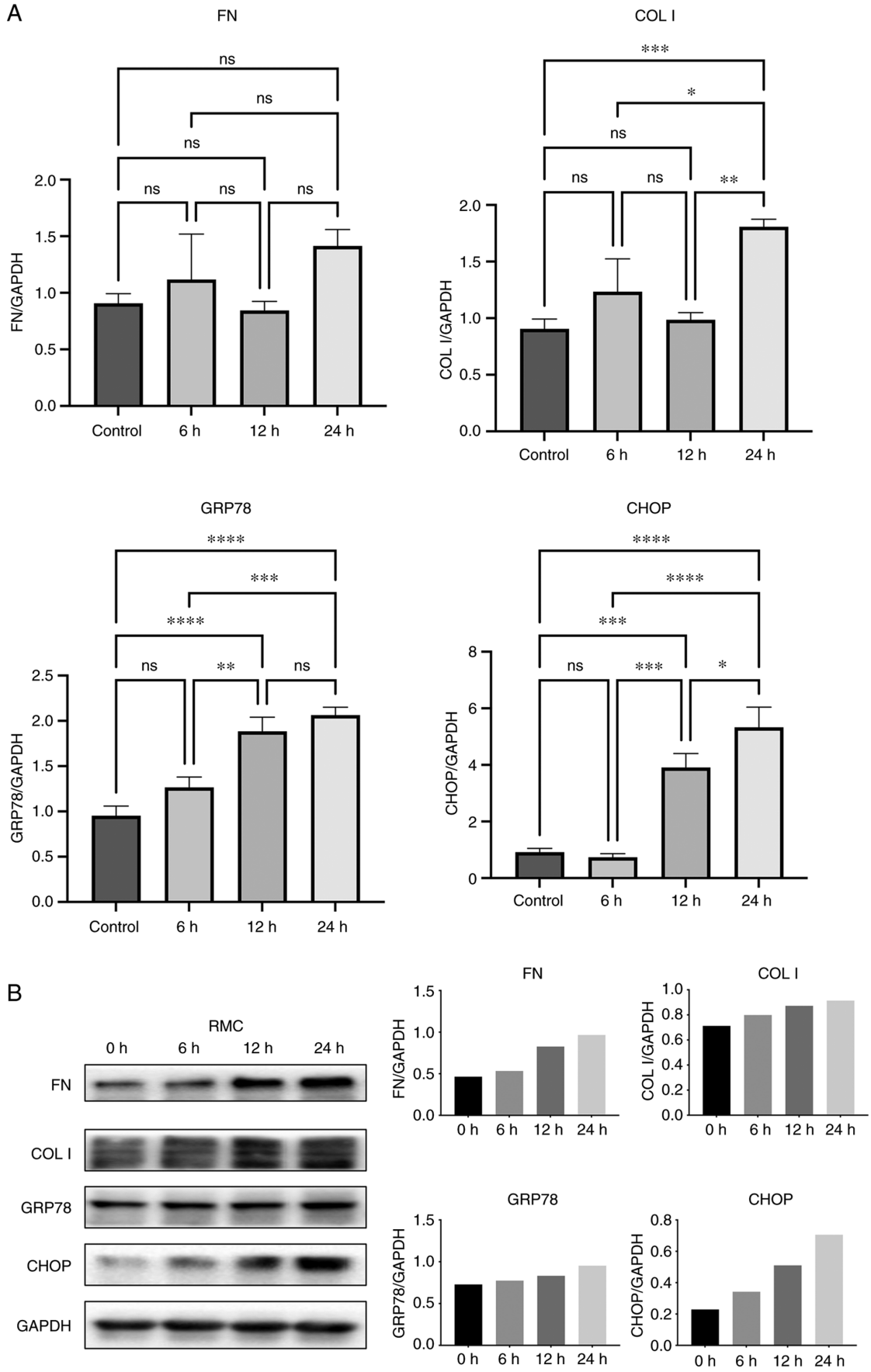

Figure 2. Effects of TGF-β 1 on the expression of FN, COL I, GRP78s and CHOP in RMCs. (A) RMCs were stimulated with TGF-β 1 (5 ng/ml) and gene expression of *FN*, *COL I*, *GRP78* and *CHOP* were assessed after 6, 12 and 24 h by RT-PCR. (B) Western blot analysis and grayscale analysis for FN, COL I, GRP78 and CHOP. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; ns, P>0.05 (not significant). RMCs, rat renal mesangial cells; CHOP; C/EBP homologous protein. FN, fibronectin; COL I, collagen I; GRP78, glucose-regulated protein 78; CHOP, C/EBP homologous protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TGF, transforming growth factor.

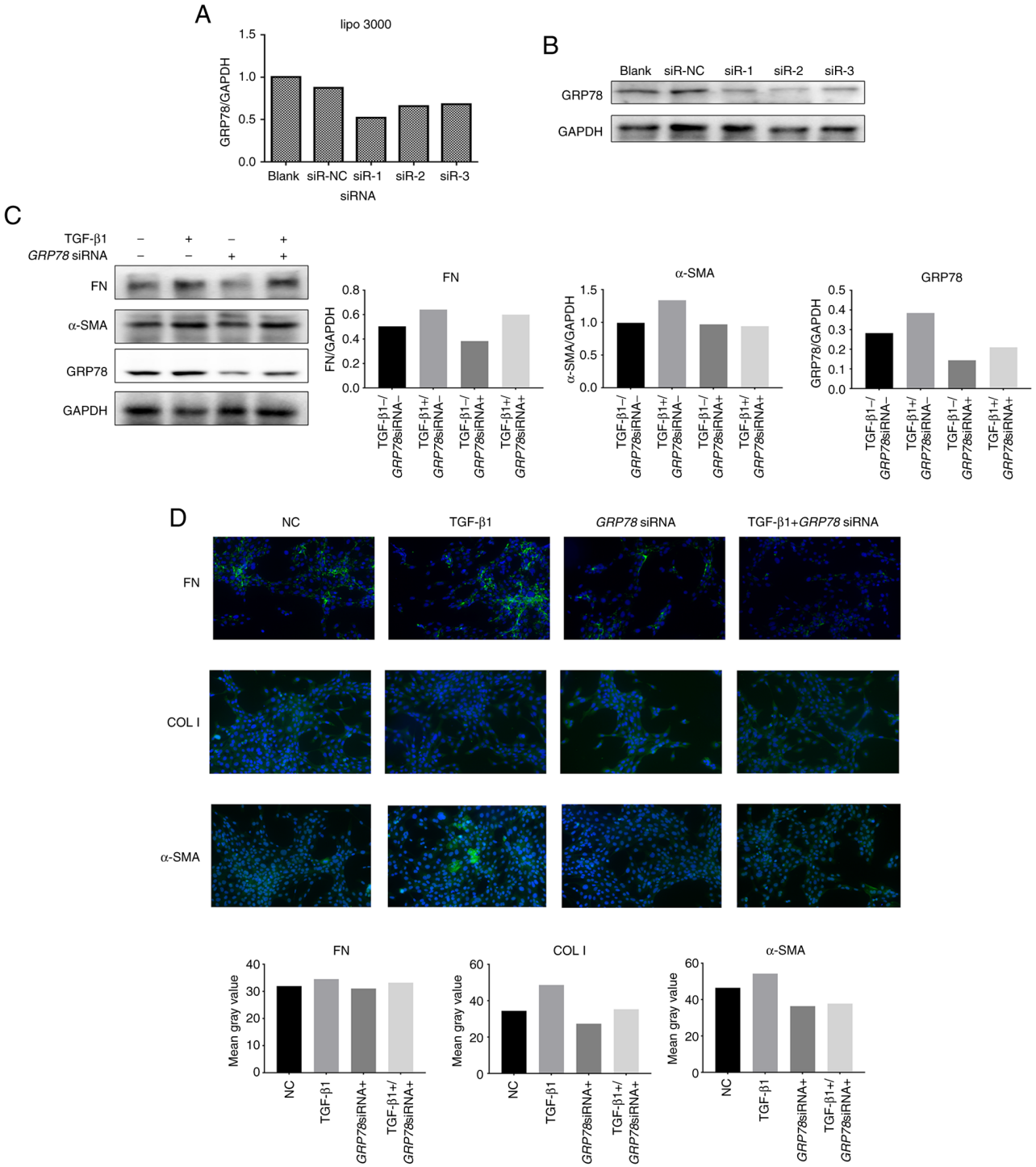

Figure 3. Silencing of *GRP78* inhibits TGF-β1-induced fibrogenesis in RMCs. (A) Three siRNAs targeting *GRP78* were individually transfected into RMCs, and the knockdown efficiency was assessed by real-time PCR at 24 h after transfection. (B) Western blot analysis. (C) RMCs were transiently transfected with one siRNA (siR-1) with the highest knockdown efficiency shown in A and then treated with (+) or without (-) TGF-β 1 (5 ng/ml, 24 h). Gene expression was measured by real-time PCR for *FN*, *α-SMA* and *GRP78*. (D) Immunofluorescence analysis of FN, COL I and α-SMA. RMCs, rat renal mesangial cells; FN, fibronectin; α-SMA, α-smooth muscle actin; GRP78, glucose-regulated protein 78; COL I, collagen I; CHOP, C/EBP homologous protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TGF, transforming growth factor.

(Fig. 4A). The TGF-β1 (5 ng/ml)-stimulated protein levels of CHOP, GRP78 and FN were higher than in the control group (Fig. 4B, the first column is the blank control group). However, TUDCA intervention markedly lowered the levels of these proteins. Furthermore, a dose-dependence experiment showed that 500 μM TUDCA produced a stronger effect than 300 or 100 μM TUDCA (Fig. 4B).

Discussion

In recent years, the growing incidence of chronic kidney disease (CKD) has significantly lowered the quality of life of afflicted individuals. Since renal fibrosis cannot be reversed and there is an absence of effective treatment, the CKD mortality rate is also increasing. Therefore, here, we explored the key regulatory

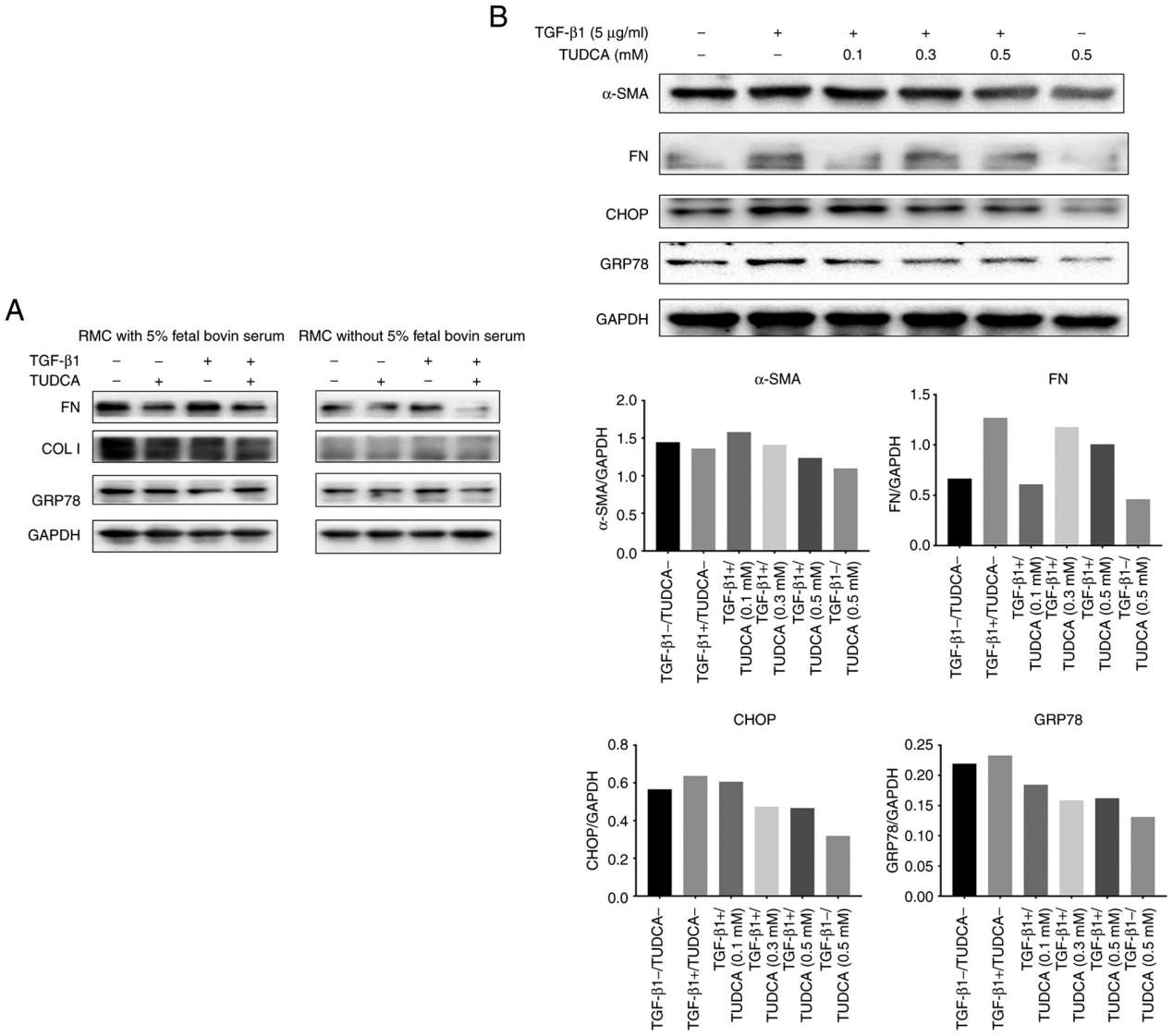

Figure 4. Effects of tauroursodeoxycholic acid (TUDCA) on fibrogenesis in RMCs. (A) RMCs were treated with TUDCA (0.1 mM) for 1 h, treated with (+) or without (-) TGF-β1 (5 ng/ml) for 24 h in medium with or without 5% fetal bovine serum, and protein expression was measured by western blot analysis for FN, COL I and GRP78. (B) RMCs were treated with TUDCA (0.1, 0.3 and 0.5 mM) for 1 h, treated with (+) or without (-) TGF-β1 for 24 h in medium without 5% fetal bovine serum, and protein expression was measured by western blot analysis for FN, α-SMA, CHOP and GRP78. FN, fibronectin; α-SMA, α-smooth muscle actin; GRP78, glucose-regulated protein 78; COL I, collagen I; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TGF, transforming growth factor.

mechanisms of renal fibrosis and used drugs to block or delay its progress. The current mainstream view is that renal fibrosis is caused by extracellular matrix (ECM) accumulation involving epithelial-mesenchymal transition (EMT), transforming growth factor (TGF)-β signal transduction, oxidative stress, and proteinuria (16). Notably, endoplasmic reticulum stress (ERS) is closely related to EMT, TGF-β signal transduction, oxidative stress, and proteinuria (9). Therefore, novel drugs targeting different intracellular ER-related pathways can essentially slow the development of renal fibrosis.

In the present study, we first determined the optimal stimulation concentration and time of TGF-β1 required for fibrosis characteristics in renal mesangial cells (RMCs). Then, siRNAs were employed to examine the effect of glucose-regulated protein 78 (GRP78) on TGF-β1-mediated fibrosis in RMCs. Furthermore, we found that tauroursodeoxycholic acid (TUDCA) downregulated GRP78 and CHOP to regulate ERS which, in turn, delayed TGF-β1-mediated renal fibrosis. We showed that siRNA targeting *GRP78* inhibited TGF-β1-induced fibrosis in RMCs suggesting a key role for ERS in fibrosis development. Interestingly, TUDCA could inhibit the expression of the ERS-related proteins GRP78 and C/EBP homologous protein (CHOP), thereby inhibiting TGF-β1-induced fibrosis in RMCs. This suggests that TUDCA could be a potential therapeutic drug for CKD renal fibrosis. In the present study, western blot analysis was used as the main analysis method. However, western blot analysis can only be used for identifying proteins qualitatively or semi-quantifying protein amounts roughly. For future research, more repetitive experiments and animal models are needed to confirm the conclusions of the present study.

Renal fibrosis is a complex process characterized by fibroblast proliferation and ECM accumulation (17,18). Mesangial cells are one of the main cell types that produce mesangial matrix (MM) components. Under pathological conditions, RMCs are activated leading to excessive proliferation and ECM secretion. This eventually causes the fibrosis of glomeruli. In

addition, mesangial cells also secrete various inflammatory factors, adhesion molecules, chemokines, and enzymes, all of which facilitate glomerular fibrosis (19,20). Several studies have shown that RMCs play an important role in the pathogenesis of glomerular fibrosis, which ultimately causes glomerular sclerosis.

TGF-β1 promotes the proliferation of mesangial cells and also significantly downregulates the ECM degradation via MMP antagonists (tissue inhibitors of metalloproteinases, TIMPs). This aggravates the accumulation of glomerular ECM, causing severe renal fibrosis, further leading to glomerular sclerosis, and ultimately worsening renal function (21,22). We showed that silencing of *GRP78* can inhibit fibrosis in RMCs. A previous study using the angiotensin II reperfusion model reported that inhibition of ERS reduced the activity of TGF-β1 which, in turn, reduced myocardial hypertrophy and fibrosis (23). This is consistent with our findings. In addition, inhibition of GRP78 in human and mouse lung fibroblasts was shown to downregulate fibrosis markers such as collagen and α-SMA (24).

Notably, in this study, inhibiting the expression of GRP78 alone could not significantly downregulate the TGF-β1-induced levels of FN and α-SMA. However, TUDCA could downregulate both GRP78 and CHOP in ERS through signaling pathways such as AGEs, which are the end-products of glycosylation and significantly inhibit the TGF-β1-induced expression of RMC fibrosis markers (FN, α-SMA). A previous study showed that downregulation of CHOP reduced unilateral ureteral ligation-induced mouse renal tubular cell apoptosis and renal fibrosis via inhibition of the HMGB1/TLR4/NFκB/IL-1β signaling pathway and downstream TGF-β1/Smad2/3 signaling (25). In addition, the absence of CHOP was found to reduce the recruitment of pro-fibrotic factors, oxidative stress, and inflammatory cells including macrophages (26). Therefore, we speculate that CHOP could be an important player in renal fibrosis involving ERS-related pathways. However, there could be other related factors/mechanisms promoting renal fibrosis that need to be further investigated to provide a theoretical basis for the development of new drugs.

In summary, we confirmed that the ERS-related transcription factor CHOP may be involved in TGF-β1-induced renal fibrosis, supporting the findings of previous studies. In addition, TUDCA, a potential drug candidate for CKD, can delay the process of renal mesangial cell fibrosis by inhibiting TGF-β1-induced accumulation of ECM.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

LL was responsible for the conceptualization of the research design, performing experiments and formal analysis and wrote the original draft. ZYG was responsible for the conceptualization of the research design, performing experiments and formal analysis. JW was responsible for the conceptualization of the research design, performing experiments, formal analysis, and writing, reviewing and editing the manuscript. PPF conducted part of the experiments and the formal analysis. YFJ was responsible for the conceptualization of the research design, and writing, reviewing and editing the manuscript. CGX was responsible for funding acquisition, conceptualization of the research design, methodology, performing experiments, and writing, reviewing and editing the manuscript. All authors have read and approved the final manuscript, and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity (in particulary the data provided) of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The study was approved by the Clinical Research Ethics Committee of Eastern Hepatobiliary Surgery Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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