Quercetin is able to alleviate TGF-β-induced fibrosis in renal tubular epithelial cells by suppressing miR-21

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Abstract. Patients with chronic kidney disease (CKD) are characterized by a gradual loss of kidney function over time. A number of studies have indicated that tubule interstitial fibrosis (TIF) is associated with the occurrence and development of CKD. The aim of the present study was to investigate the effect of quercetin treatment on the fibrosis of renal tubular epithelial cells and to determine whether the anti-fibrotic effects of quercetin are achieved via microRNA (miR)-21. Human tubular epithelial HK-2 cells were cultured with transforming growth factor (TGF)-β to induce fibrosis and the expression of fibrotic markers collagen I, fibronectin, α-smooth muscle actin (SMA) and epithelial-cadherin were measured using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting. Cells were treated with 7.5, 15 or 30 mg/ml quercetin, following which fibrosis and miR-21 expression were evaluated. Quercetin-treated cells were transfected with miR-21 mimics and the expression of fibrotic markers was examined using RT-qPCR. Finally, the expression of fibrosis-associated miR-21 target genes, phosphatase and tensin homolog (PTEN) and TIMP Metallopeptidase Inhibitor 3 (TIMP3), was measured in cells treated with quercetin with or without miR-21 mimics using RT-qPCR, western blotting and immunocytochemistry. The results revealed that TGF-β treatment induced a significant increase in the expression of fibrotic markers in HK-2 cells, while quercetin treatment partially inhibited the fibrosis of HK-2 cells. Furthermore, quercetin treatment significantly inhibited TGF-β-induced miR-21 upregulation and transfection with miR-21 mimics reversed the anti-fibrotic effects of quercetin. Quercetin treatment markedly upregulated PTEN and TIMP3 expression, whereas transfection with miR-21 mimics reversed this effect. The results of the present study suggest that quercetin is able to alleviate TGF-β-induced fibrosis in HK-2 cells via suppressing the miR-21 and upregulating PTEN and TIMP3. Quercetin may have potential as an anti-fibrotic treatment for patients with renal fibrosis.

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Introduction

Chronic kidney disease (CKD) is the final stage of a number of renal diseases. In recent years, it has been reported that tubule interstitial fibrosis (TIF) is associated with the occurrence and development of CKD (1). Tubular epithelial cells (TECs) serve important roles in maintaining renal function and the progression of TIF (2). Kidney injury may lead to the phenotypic transformation of TECs (3), which is similar to the process of epithelial mesenchymal transition (EMT). Under such conditions, TECs acquire some properties of fibroblasts, producing excessive extracellular matrix (ECM) and related proteins, including collagen and fibronectin, leading to fibrosis of the kidney cells and eventual nephropathy (4,5).

The pathogenesis of TIF remains unclear. It has been reported that transforming growth factor (TGF)-β serves a central role in the onset and progression of kidney fibrosis (6-8). MicroRNAs (miRNAs or miRs) are a family of small non-coding RNAs 18-25 nucleotides in length (9). miRNAs are able to bind to the 3'-untranslated regions (3'-UTR) of their paired mRNAs to suppress the expression of target genes (10). A number of miRNAs have been reported to participate in the pathogenesis of many diseases, including cancers, autoimmune diseases, cardiovascular diseases and acute and chronic renal disease (10-12). However, the underlying mechanisms of miRNAs in renal diseases require further investigation.

Quercetin is a widely available flavonoid that can be isolated from many plants, vegetables and fruits (13). Increasing evidence has indicated that quercetin exerts various biological activities, such as antioxidant, anti-tumor, anti-angiogenesis and anti-inflammatory functions (14,15). Quercetin has also been reported to have a therapeutic effect on renal function and tissue damage (16,17). To the best of our knowledge, the effect of quercetin on kidney fibrosis and whether miRNAs are serving a role in quercetin-induced renal protective effects has not been reported.

A previous study reported that the TGF-β1 signaling pathway is vital for the induction of renal fibrosis (18). In the present study, in order to simulate renal fibrosis *in vitro*, the human tubular epithelial cell line HK-2 was treated with TGF-β according to previous reports (19,20). The role of miR-21 in mediating the renal protective effect of quercetin was assessed using TGF-β-induced fibrosis in HK-2 cells to provide a theoretical basis for the treatment of nephropathy using quercetin.

Materials and methods

Cell culture and treatments. The human tubular epithelial cell line HK-2 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured according to the manufacturer's protocols. Cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and streptomycin in a humidified incubator at 37˚C with 5% CO_2. Fibrosis was induced by treatment with 100 ng/ml TGF-β for 72 h. The quercetin groups were treated with medium containing 7.5, 15 or 30 mg/ml quercetin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

Cell proliferation detection. Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) was used according to the manufacturer's protocol to assess cell proliferation. Briefly, HK-2 cells were treated with 7.5, 15 or 30 mg/ml quercetin for 24, 48 and 72 h. Cells were subsequently incubated with 10 μl/well CCK8 at 37˚C for 4 h. Finally, the optical density (OD) at 450 nm was measured using a multi-well spectrophotometer (Thermo Fisher Scientific, Inc.).

Cell transfection. miR-21 inhibitor and miR-21 mimic oligonucleotides (3'-AGUUGUAGUCAGACUAUUCGAU-5') were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). Cells were transfected with miR-21 inhibitor (100 nM) or miR-21 mimics (50 nM) using Lipofectamine® RNAi Max (Thermo Fisher Scientific, Inc.). At 48 h post transfection, cells were trypsinized and harvested (800 x g, 5 min, 4˚C) for future analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). RT-qPCR was performed with an SYBR ExScript RT-PCR kit (Takara Bio, Inc., Dalian, China) on an ABI 7500 Real-Time PCR System machine (Applied Biosystems; Thermo Fisher Scientific, Inc.). Reverse transcription reaction was performed for 1 h at 37˚C followed by 5 min at 95˚C. The thermocycling conditions for the qPCR step were as follows: 95˚C for 30 sec followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) with sequences as follows: Collagen I forward, 5'-TGGCCAAGAAGACATCCCTGAAGT-3' and reverse, 5'-ACATCAGGTTTCCACGTCTCACCA-3'; fibronectin forward, 5'-CCATCGCAAACCGCTGCCAT-3' and reverse, 5'-AACACTTCTCAGCTATGGGCTT-3'; GAPDH forward, 5'-AAGAAGGTGGTGAAGCAGGC-3' and reverse, 5'-TCCACCACCCTGTTGCTGTA-3'; α-smooth muscle actin (α-SMA) forward, 5'-ACTGAGCGTGGCTATTCCTCCGTT-3' and reverse, 5'-GCAGTGGCCATCTCATTTTCA-3'; epithelial E-cadherin forward, 5'-TACACTGCCCAGGAGCCAGA-3' and reverse; 5'-TGGCACCAGTGTCCGGATTA-3'; phosphatase and tensin homolog (PTEN) forward, 5'-CAAGATGATGTTTGAAACTATTCCAATG-3' and reverse, 5'-CCTTTAGCTGGCAGACCACAA-3'; TIMP metallopeptidase inhibitor 3 (TIMP3) forward, 5'-CTGCAAGGGCTGGGCATC-3' and reverse, 5'-TCCATGGCCCGGTTGGCAGTGTGGAG-3'; U6 forward, 5'-ATAAGGATCCGGTCTCGCTATGAGGGCCTATTTCCCATG-3' and reverse, 5'-ATAATGTACAGGTCTCCCATGTAACTTGCTATTTCTAGCTC-3'. The relative expression of collagen I, fibronectin, SMA, (E)-cadherin, PTEN and TIMP3 in each sample was normalized to the expression of GAPDH using the $2^{-\Delta\Delta Cq}$ method (21). miR-21 expression was examined using a Hairpin-it™ miRNAs qPCR Quantitation kit (Shanghai GenePharma Co., Ltd.) and U6 was used for normalization. Primers, forward 5'-GCCGCTAGCTTATCAGACTGATGT-3' and reverse 5'-GTGCAGGGTCCGAGGT-3', were supplied by Sangon Biotech Co., Ltd.

Western blotting. Cells were lysed using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China). Bradford assays were used to determine the concentration of total extracted protein. Total proteins (40 μg) were loaded and separated on 10% SDS-PAGE gels. Proteins were transferred onto polyvinylidene fluoride membranes, blocked in 5% non-fat milk for 2 h at room temperature and incubated with the following primary antibodies: Anti-collagen I (1:1,000; cat. no. 84336; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-fibronectin (1:200; cat. no. ab2413; Abcam, Cambridge, MA, USA), anti-α-SMA (1:1,000; cat. no. 19245; Cell Signaling Technology, Inc.), anti-E-cadherin (1:1,000; cat. no. 3195; Cell Signaling Technology, Inc.), anti-PTEN (1:1,000; cat. no. 9188; Cell Signaling Technology, Inc.), anti-TIMP3 (1:1,000; cat. no. 5673; Cell Signaling Technology, Inc.) and anti-β-actin (1:1,000; cat. no. 4970; Cell Signaling Technology, Inc.) overnight at 4˚C. The membranes were subsequently washed three times with TBST buffer and incubated with the secondary horseradish peroxidase-linked antibody goat anti-rabbit IgG (1:1,000; cat. no. A0208; Beyotime Institute of Biotechnology) for 1 h at room temperature, following which they were washed three times with TBST buffer, incubated with BeyoECL Plus (Beyotime Institute of Biotechnology) and visualized using ChemiDoc™ XRS+ System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). β-actin was used as the internal control.

Immunocytochemistry (IHC). For IHC, cells were fixed in 4% paraformaldehyde overnight at room temperature and permeabilized with 0.1% Triton X-100 in 0.5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature. Cells were incubated with the rabbit anti-human PTEN (1:125; cat. no. 9188; Cell Signaling Technology, Inc.) primary antibody overnight at 4˚C. Following washing for three times with PBS, cells were incubated with Alexa Fluor® 488-conjugated rabbit mAb (1:800; cat. no. 9854; Cell

Signaling Technology, Inc.) secondary antibody for 30 min at 37˚C. Cells were visualized using a fluorescent microscope (magnification, x400).

Statistical analysis. Statistical analysis was performed using SPSS 19.0 (IBM Corp., Armonk, NY, USA). Data are presented as the mean ± standard deviation and a two-independent sample t-test was performed used to make comparisons between two groups. One-way analysis of variance followed by Dunnett's test was used for multiple group comparisons. P<0.05 was considered to indicate a significant difference.

Results

TGF-β treatment induces fibrosis in HK-2 cells. HK-2 cells were treated with TGF-β and the expression of fibrotic markers was evaluated using RT-qPCR and western blotting. Compared with untreated cells, TGF-β induced a significant increase in the expression of fibrotic markers collagen I, fibronectin, α-SMA and E-cadherin at the mRNA (Fig. 1A) and protein levels (Fig. 1B and C), suggesting that TGF-β treatment induces a fibrosis-like state in HK-2 cells *in vitro*.

Quercetin alleviates TGF-β-induced fibrosis in HK-2 cells. To assess whether quercetin is able to alleviate TGF-β-induced fibrosis in HK-2 cells, TGF-β-stimulated HK-2 cells were treated with different concentrations of quercetin and the expression of fibrotic markers was evaluated using RT-qPCR and western blotting. CCK-8 results revealed that treatment with 7.5 and 15 mg/ml quercetin had a limited effect on cell growth, while 30 mg/ml quercetin had a slight cytotoxic effect (Fig. 2A). Compared with the TGF-β group, TGF-β + 7.5 mg/ml quercetin induced a significant decrease in the expression of collagen I, fibronectin and α-SMA. In the TGF-β + 15 mg/ml and TGF-β + 30 mg/ml quercetin groups, a significant decrease in collagen I, fibronectin, α-SMA and E-cadherin mRNA expression was observed (Fig. 2B). Furthermore, quercetin induced a significant decrease in the protein expression of fibrotic markers (Fig. 2C). The effect of quercetin on α-SMA and fibronectin expression was dose-dependent (Fig. 2C). Based on these results, 15 mg/ml quercetin had been selected for further investigations.

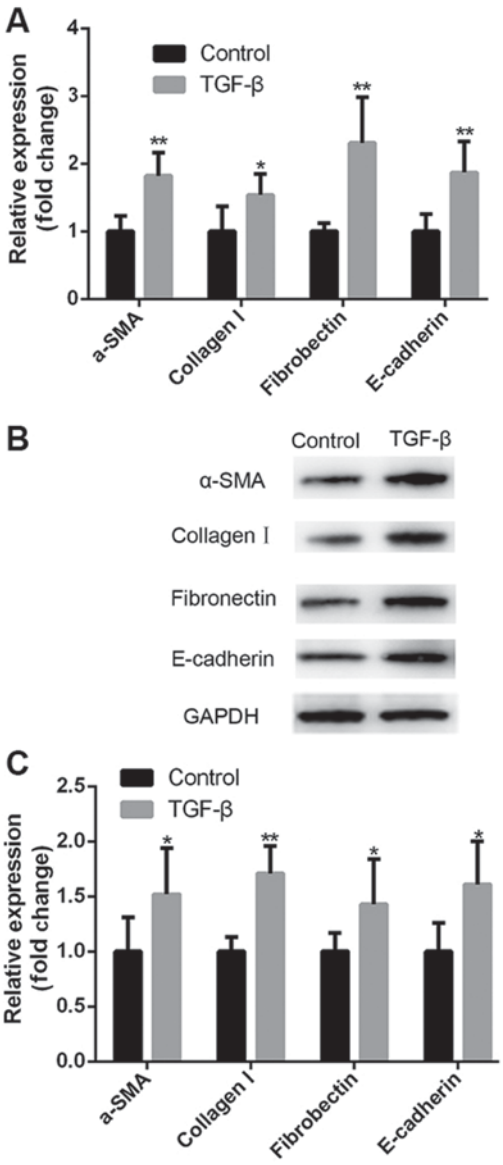

Figure 1. TGF-β treatment induces a fibrosis-like state in HK-2 cells *in vitro*. Relative expression of fibrotic markers α-SMA, collagen I, fibronectin and E-cadherin at the (A) mRNA and (B) protein levels. (C) Quantified results of western blotting. *P<0.05 and **P<0.01 vs. Control. TGF-β, transforming growth factor-β; α-SMA, α-smooth muscle actin; E-cadherin, epithelial cadherin.

Quercetin alleviates TGF-β induced fibrosis via regulating miR-21 expression. The role of miR-21 in quercetin-induced anti-fibrosis was investigated. TGF-β treatment induced a significant increase in miR-21 expression, while quercetin treatment decreased the TGF-β-induced miR-21 upregulation in HK-2 cells (Fig. 3A). Transfection with miR-21 mimics inhibited the quercetin-induced anti-fibrotic effects, increasing the expression of collagen I, fibronectin, α-SMA and E-cadherin at the mRNA (Fig. 3B) and protein levels (Fig. 3C and D).

Quercetin increases the expression of miR-21 target genes in TGF-β treated HK-2 cells. PTEN and TIMP3 have previously been confirmed as direct targets of miR-21 (22,23). To further explore the mechanism of miR-21 in quercetin-induced anti-fibrotic effects, the expression of PTEN and TIMP3 in untreated fibrotic and quercetin-treated fibrotic HK-2 cells was assessed. Quercetin treatment induced a significant increase in the expression of PTEN and TIMP3 at the mRNA (Fig. 4A) and protein levels (Fig. 4B-D). Furthermore, transfection with miR-21 mimics partially inhibited the quercetin-induced increase in PTEN and TIMP3 expression (Fig. 4A-C).

Discussion

The renal protective effect of quercetin has been discussed in a number of studies. Gelen *et al* (24) reported that quercetin protected renal function in a rat model of obesity, while Yuksel *et al* (25) demonstrated that quercetin was able to reduce methotrexate-induced oxidative stress in rat models, suggesting that quercetin can alleviate methotrexate-induced

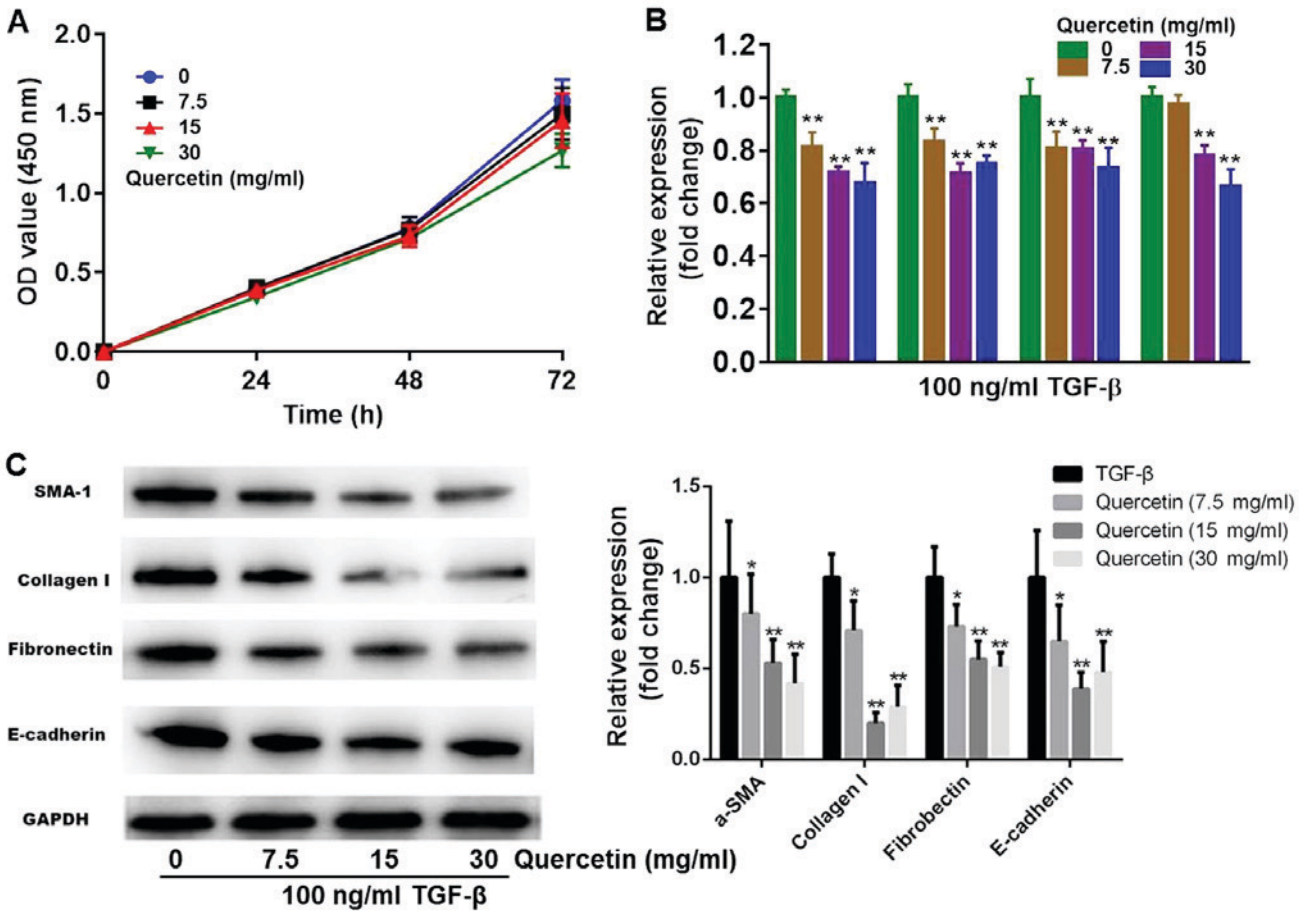

Figure 2. Quercetin alleviates TGF-β-induced fibrosis in HK-2 cells. (A) HK-2 cells were treated with 7.5, 15 or 30 mg/ml quercetin for 24, 48 or 72 h and the OD was measured at 450 nm. Relative expression of fibrotic markers α-SMA, collagen I, fibronectin and E-cadherin at the (B) mRNA and (C) protein levels. *P<0.05 and **P<0.01 vs. Control. TGF-β, transforming growth factor-β; OD, optical density; α-SMA, α-smooth muscle actin; E-cadherin, epithelial cadherin.

renal damage and restore renal function. Elbe *et al* (26) revealed that quercetin is able to reduce ciprofloxacin-induced oxidative stress in rats, suggesting that quercetin may be an effective treatment for injuries. In the present study, fibrosis was induced in HK-2 cells using TGF-β and it was demonstrated that treatment with 7.5, 15 or 30 mg/ml quercetin induced a significant decrease in the expression of fibrotic markers. These results suggest that the anti-fibrotic effect of quercetin may not be dose-dependent, as no significant difference was observed in the expression of fibrotic markers between the 15 and 30 mg/ml groups. Taken together, these results indicate that treatment with an appropriate dosage of quercetin may effectively alleviate TGF-β-induced fibrosis in HK-2 cells *in vitro*.

miR-21 has previously been reported to be a profibrotic microRNA. miR-21-5p expression is associated with the incidence of renal fibrosis in patients with IgA nephropathy (27), while an *in vitro* study revealed that miR-21 may serve a role in the pathogenesis of TIF by targeting DDAH1 (28). Furthermore, Wang *et al* (29) demonstrated that miR-21 overexpression can cause renal fibrosis via the MMP9/TIMP1 signaling pathway in mice, suggesting that miR-21 has potential as a novel therapeutic target for the management of diabetic nephropathy. In the present study, it was demonstrated that miR-21 was upregulated in fibrosis in HK-2 cells, which is consistent with previous findings. It was also observed that quercetin treatment induced a significant decrease in the expression of miR-21, suggesting that miR-21 may serve a role in the mechanism by which quercetin induces anti-fibrotic effects. Furthermore, transfection with miR-21 mimics partially inhibited quercetin-induced anti-fibrotic effects. These results suggest that the anti-fibrotic effects of quercetin may be achieved in part by downregulating miR-21, a profibrotic factor.

PTEN and TIMP3 are known to be anti-fibrotic factors (30-32) and are downregulated in fibrotic renal tissues. Downregulation of either PTEN (29) or TIMP3 (31) may enhance the degree of TIF. Bioinformatics has been used to predict PTEN and TIMP3 as direct targets of miR-21, which has been confirmed using dual-luciferase reporter assays (32,33). To further explore the mechanism of miR-21 in quercetin-induced anti-fibrotic effects, the expression of PTEN and TIMP3 was compared between quercetin-treated and untreated fibrotic HK-2 cells. The results indicated that quercetin treatment induced a significant increase in the expression of PTEN and TIMP3 at the mRNA and protein levels, while transfection with miR-21 mimics partially inhibited the quercetin-induced PTEN and TIMP3 upregulation. These results suggest that quercetin downregulates miR-21 and upregulates anti-fibrotic gene expression, thereby

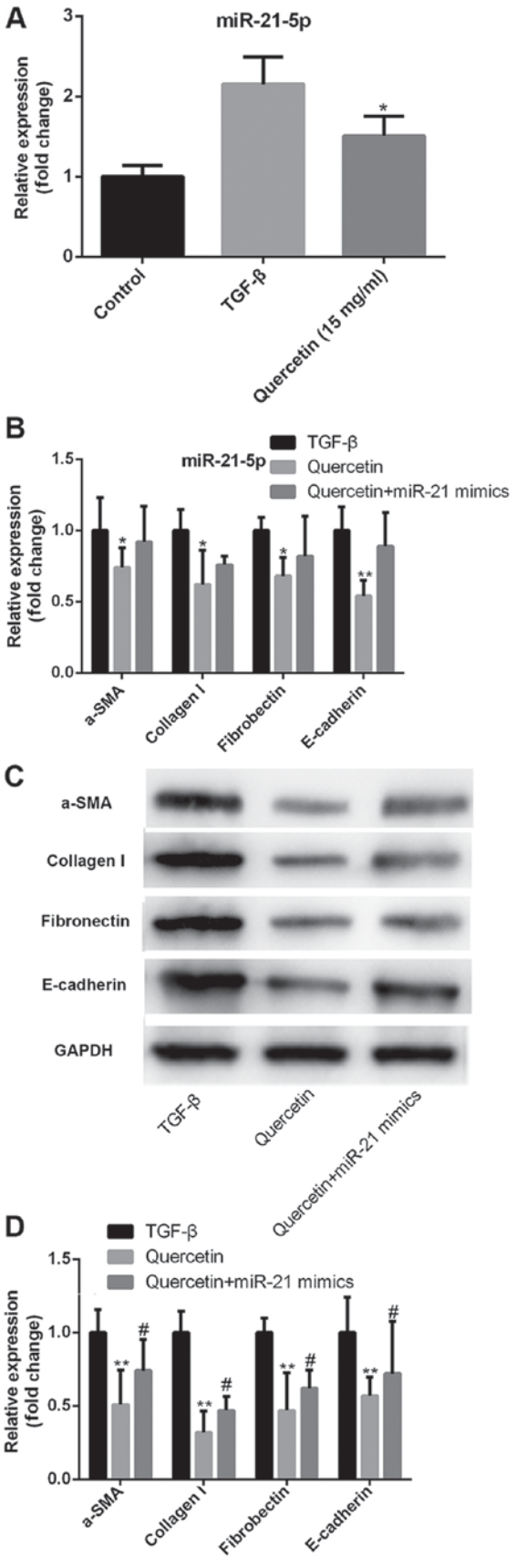

Figure 3. Quercetin alleviates TGF-β-induced fibrosis by regulating the expression of miR-21. (A) Relative expression of miR-17-5p mRNA. Relative expression of fibrotic markers α-SMA, collagen I, fibronectin and E-cadherin at the (B) mRNA and (C) protein levels. (D) Quantified results of western blotting. *P<0.05 and **P<0.01 vs. TGF-β; #P<0.05 vs. quercetin. TGF-β, transforming growth factor-β; miR, microRNA; α-SMA, α-smooth muscle actin; E-cadherin, epithelial cadherin.

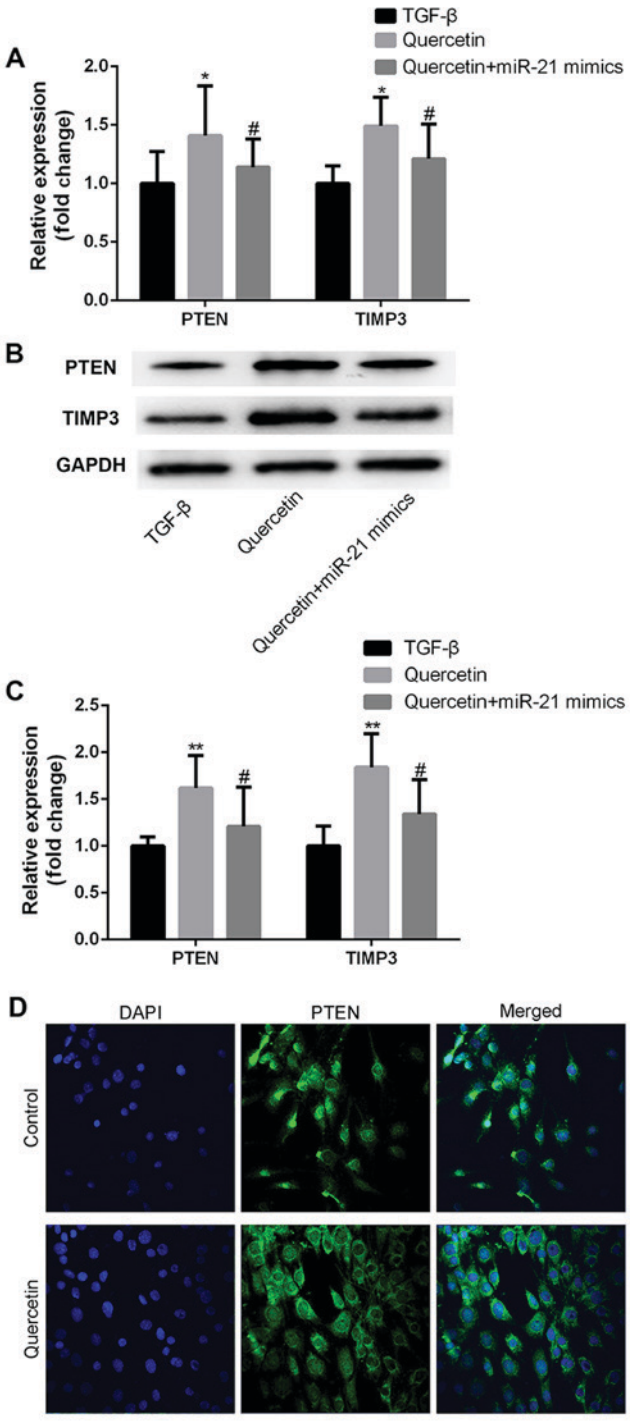

Figure 4. Quercetin increases the expression of miR-21 target genes in TGF-β-treated HK-2 cells. Relative expression of PTEN and TIMP3 at the (A) mRNA and (B) protein levels. (C) Quantified results of western blotting. (D) Immunohistochemistry results for PTEN expression. *P<0.05 and **P<0.01 vs. TGF-β; #P<0.05 vs. quercetin. miR, microRNA; TGF-β, transforming growth factor-β; PTEN, phosphatase and tensin homolog; TIMP3, TIMP Metallopeptidase Inhibitor 3.

inhibiting the progression of kidney fibrosis and protecting renal function.

The present study is not without limitations. Only cellular experiments were performed; in future studies, the roles of quercetin and miR-21 should be further explored using animal models. In conclusion, the results of the present study suggest that quercetin is able to alleviate TGF-β-induced

fibrosis in tubular epithelial cells by suppressing miR-21. These findings provide a novel insight into the effects of quercetin as an anti-fibrotic drug for the treatment of renal fibrosis.

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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 on reasonable request.

Authors' contributions

YCao and JH contributed to conception and design of the study. YCao, JH and JS performed the experiments. LJ conducted data acquisition, analysis and interpretation. YCong was involved in data analysis, interpretation and manuscript development. GR guided the experimental design, reviewed the manuscript and supervised the study. All authors read and approved the final version of this manuscript and agreed to be accountable for all aspects of this study.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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