Regulation of CTGF Expression by miR-133b for the Treatment of Renal Interstitial Fibrosis in Old UUO Rats

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Research

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Abstract

Introduction: Renal interstitial fibrosis, an important pathological feature of kidney aging and chronic renal failure, is regulated by mesenchymal stem cells (MSCs). We have previously demonstrated the high expression of miR-133b in MSC-derived extracellular vesicles (MSC-EVs) from old rats, which mediated the inhibition of epithelial-mesenchymal transition (EMT) of renal tubules induced by transforming growth factor-β1 (TGF-β1). We investigated the effect of miR-133b for the treatment of geriatric renal interstitial fibrosis and evaluated its target genes.

Methods: miR-133b expression induced during the EMT of HK2 cells by TGF-β1 at different concentrations (0, 6, 8, and 10 ng/mL) and time points (0, 24, 48, and 72 h) was detected using real-time polymerase chain reaction. The target genes of miR-133b were validated using a dual-luciferase reporter assay. In vitro experiments were performed to observe mRNA and protein expression of miR-133b targets, E-cadherin, α-smooth muscle actin (SMA), fibronectin, and collagen 3A1 (Col3A1), in HK2 cells transfected with miR-133b under TGF-β1 stimulation. A 24-week-old unilateral ureteral obstruction (UUO) mouse model was established and injected with transfection reagent and miR-133b into the caudal vein. miR-133b target gene and other indexes mentioned above mRNA and protein levels and renal interstitial fibrosis were detected at 7 and 14 days.

Results: miR-133b expression gradually decreased with an increase in TGF-β1 concentration and treatment time, and miR-133b mimic downregulated connective tissue growth factor (CTGF) expression. Dual-luciferase reporter assay confirmed CTGF as a direct target of miR-133b. miR-133b mimic transfection inhibited the TGF-β1-induced EMT of HK2 cells; this effect was reversed by CTGF overexpression. miRNA-133b expression significantly increased (approximately 70-100 times) in mouse kidneys after injection of the miRNA-133b overexpression complex, significantly alleviating renal interstitial fibrosis in UUO mice.

Conclusion: miR-133b exerted targeted inhibitory effects on CTGF expression, consequently reducing the TGF-β1-induced EMT of HK2 cells and renal interstitial fibrosis in old UUO mice.

Background

Aging is characterized by significant changes in the structure and function of the kidney, even in the absence of age-related comorbidities. Renal interstitial fibrosis is an important pathological feature of kidney aging [1], and bone marrow mesenchymal stem cells (MSCs) play an important role in the regulation of renal interstitial fibrosis. Transplantation of the bone marrow from young mice into old mice was shown to significantly reduce renal fibrosis as well as the expression of aging markers in the recipient mice; bone marrow cells did not directly replace parenchymal cells but instead exerted paracrine effects on renal parenchymal cells [2]. Further, the injection of MSCs and MSC-derived extracellular vesicles (MSC-EVs) was shown to significantly alleviate renal interstitial fibrosis in a unilateral ureteral obstruction (UUO) model [3, 4].
Aging can significantly alter the number of stem cells and their regenerative capacity and functions [5]. Our previous research revealed the significant differences in the expression profiles of microRNAs (miRNAs) of MSC-EVs derived from the bone marrow of young and old rats. We found that miR-133b-3p was highly expressed in the MSC-EVs of old rats and inhibited the epithelial-mesenchymal transition (EMT) of renal tubular cells induced by transforming growth factor (TGF)-β1 [6]. The inhibitory effects of MSC-EVs against renal fibrosis decreased with age. miR-133 of MSC-EVs derived from old rats was thought to exhibit important intervening effects on renal fibrosis [7].

miR-133 ameliorates cardiac fibrosis [8–10], reduces the TGF-β1-mediated EMT of bladder smooth muscle epithelial cells [11], and directly targets the connective tissue growth factor (CTGF) [9, 11]. Whether CTGF is a target involved in the miR-133-mediated inhibition of the EMT of renal tubular epithelial cells is still unclear, and studies are warranted to investigate the effect of transfection with exogenous miR-133 in geriatric renal interstitial fibrosis.

Here, the EMT of the human renal proximal tubular epithelial cell line HK2 was stimulated by TGF-β1 in vitro to investigate the role of miR-133b and its target genes in this process. A UUO model was established using old C5BL/6J7 mice (aged 24 months) intravenously injected with an miR-133b transfection complex to verify the effect of miR-133b overexpression in geriatric renal fibrosis.

**Methods**

**TGF-β1 stimulation and miR-133b transfection**

The HK2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Corning, USA) supplemented with 5% fetal bovine serum. After reaching 50% confluency, the cells were synchronized in serum-free DMEM/F12 for 18 h and then stimulated with TGF-β1 at 6, 8, and 10 ng/mL concentrations for 24, 48, and 72 h. In the transfection experiment, miR-133b mimic and miRNA mimic control (GenePharma, China) were transfected into HK2 cells for 6 h, as per the instructions of jetPRIME® transfection reagent (Polyplus transfection, France). Following transfection, the cells were cultured in DMEM/F12 with 5% serum for 18 h and then incubated with DMEM/F12 with 5% serum and 8 ng/mL TGF-β1 (PeproTech, USA) for 48 h.

**RNA extraction and real-time polymerase chain reaction (PCR)**

Total RNA was extracted from HK2 cells and kidney tissues of each group by Trizol, and used to synthesize miR-133b and U6 cDNA using miScript II RT Kit (QIAGEN, China). Primers specific for target genes were designed with reference to their mRNA-coding regions in GenBank using Primer 5.0 software. The primer sequences were verified on BLAST. The total RNA was used to synthesize cDNA of target genes using ReverTra Ace qPCR RT Master Mix kit (TOYOBO, Japan). The expression of the genes encoding miR-133b, CTGF, E-cadherin, α-smooth muscle actin (SMA), fibronectin, collagen 3A 1 (Col3A1), U6, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected on an ABI-prism-7500
sequence detection system (Applied Biosystems, USA) using miScript SYBR Green PCR Kit (QIAGEN). The relative expression level was calculated using U6 or GAPDH as internal control.

hsa-miR-133b 5’TTTGGTCCCTTCAACCAGCTA 3’; 
mmu-miR-133b 5’ TTTGGTCCCTTCAACCAGCTA 3’;

hsa-CTGF 5’ GGCCTTTCTTGTGACTTCG3’ and 5’ ATGCAGGGAGCACCATCT 3’; 
has-α-SMA 5’ ACTGCAGCATCTCATCC3’ and 5’CCCATCAGGAAACTCGTAA3’; 
has-Ecad 5’ CTGAGAACGAGGCTAACG3’ and 5’AGAACGAGGCTAACG3’; 
has-FN 5’ GTGCCACCTACAACATC3’ and 5’CCACGGTAACCACTCCTT3’; 
has-Col3A1 5’ CTTCTCGCTGCTTCTC3’ and 5’CTATCCGCATAGGAGCCT 3’;

Western blot analysis

After lysis and denaturation of HK2 cells or kidney tissues from each group using radioimmunoprecipitation assay (RIPA) buffer, the proteins (50 µg) were separated on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto nitrocellulose (NC) membranes. After blocking with 1 × casein for 1 h to prevent non-specific binding, NC membranes were incubated with the following primary antibodies at 4 °C for overnight: (a) Rabbit monoclonal antibody E-cadherin (BD Bioscience, USA) diluted to 1:100; (b) rabbit monoclonal antibody against α-SMA (Abcam, UK) diluted to 1:300; (c) mouse monoclonal antibody against CTGF (Abcam) diluted to 1:200; (d) rabbit polyclonal antibody (Proteintech, USA) against fibronectin diluted to 1:500; (e) rabbit polyclonal Col3A1 antibody (Proteintech) diluted to 1:500; (f) mouse monoclonal β-actin antibody (Beyotime, China) diluted to 1:10000. The membranes were washed with TBST (Tris buffered saline, with Tween-20, 20 mM of Tris, 140 mM of NaCl, and 0.1% Tween-20) and probed with 1:1000 diluted secondary antibodies at room temperature (25°C) for 2 h. Enhanced chemiluminescence (ECL) western blotting kit (APPLYGEN, China)
was used to detect target bands, and β-actin was used as internal reference to calculate the relative expression of protein in each experimental group.

**Prediction of target genes of miR-133b**

The target genes of miR-133b were predicted using three commonly employed target gene prediction software, namely TargetScan (http://www.targetscan.org/), miRBase (http://www.mirbase.org/), and PiTar (http://genie.weizmann.ac.il/index.html).

**Dual-luciferase reporter assay**

The seed sequence for the binding between CTGF and miR-133b was searched using the bioinformatic software TargetScan. The sequence 5′-AUUUGUUGAGUGUGGAAGG-3′ containing the 3′-untranslated region (UTR) of CTGF was synthesized and cloned into a luciferase reporter vector GP-miRGLO (GenePharma), which was termed as miRGLO-Wt-CTGF. A mutant sequence 5′-AUUUGUUGAGUGUGGAUAA-3′ of the target was also synthesized and cloned into the plasmid to obtain miRGLO-Mut-CTGF, which was used as the negative control.

293T cells from logarithmic growth phase were digested with pancreatin and seeded in 48-well plates for 24 h. After reaching 80% confluency, the cells were transfected the cell fusion reagent. According to the instructions of jetPRIME® transfection reagent, the synthesized miR-133b and miRNA mimic control (NC-miR) were respectively co-transfected with miRGLO-Wt-CTGF or miRGLO-Mut-CTGF into 293T cells. After 48 h, the cells were lysed using a passive lysis buffer (Promega, USA) and the cell lysate collected. The luciferase activity of the lysate was detected according to the steps indicated in the dual-luciferase reporter assay system (Promega).

**Immunofluorescence staining**

HK2 cells were seeded at about 10^5 cells/well in six-well plates with glass cover slips and disinfected at high temperature and high pressure. The plates were placed in an incubator at 37 °C with 5% carbon dioxide for 6 h to allow the cells to adhere to the glass cover slips. After synchronization and transfection as mentioned above, the cells were incubated with DMEM/F12 containing 5% serum with or without 8 ng/mL TGF-β1 for 48 h. The cells were then fixed with 4% paraformaldehyde at room temperature for 20 min and treated with 0.2% Triton X-100 for 2 min for permeabilization. The cells were blocked with 5% bovine serum albumin (BSA) at room temperature for 1 h and then treated with anti-E-cadherin rabbit monoclonal primary antibody (1:100 dilution) and α-SMA antibody (1:100 dilution) diluted in 5% BSA at 4 °C for overnight. After washing with phosphate-buffered saline (PBS), the cells were probed with an anti-rabbit fluorescein isothiocyanate (FITC)-conjugated fluorescent secondary antibody (Beyotime) (1:400 dilution) at room temperature in the dark for 2 h. The slides were then washed with PBS and treated with 4′,6-diamidino-2-phenylindole (DAPI) (ZSGB-BIO, China) fluorescence nuclear staining mounting medium. The expression of α-SMA and E-cadherin in HK2 cells from each group was observed under a fluorescence microscope (100×) with random fields of vision.

**Experimental animals and establishment of a UUO model**
Animal care and experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee of Chinese PLA General Hospital. A total of 28 female C5BL/6J7 mice (SPF grade), aged 24 months, weighing 20 ± 2 g, were provided by SPF (Beijing) Biotechnology Co., Ltd. Mice were randomly divided into sham (n = 8), UUO + NS-miR-133b (n = 10), and UUO + miR-133b groups (n = 10).

To establish a UUO model, each mouse was anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg) and the abdominal cavity was opened under sterile conditions. The left ureter was dissociated, double-ligated, and disconnected at 15 mm below the renal pelvis with 4 - 0 thread. The abdominal cavity was closed by layered suture. In the control group, the abdominal cavity was closed immediately after the ureter was dissociated.

miR-133 or NC-miR (3 mg/kg) was diluted to 1 µg/µL concentration using endotoxin-free purified water. The transfection reagent Entranster-\textit{in vivo} (Engreen, China) was diluted in a 10% glucose solution at a final glucose concentration of 5%. The transfection complex was prepared by mixing the two agents and incubating for 15 min. The UUO + miR-133b and UUO + NC-miR groups were administered with miR-133b and NC-miRNA transfection complex (100 µL/animal), respectively, by a caudal vein injection at 24 h before operation and once every 3 days thereafter. The sham group was given the same amount of normal saline by caudal vein injection. The mice were sacrificed at 7 and 14 days after UUO (4 mice from the sham group and 5 mice from the UUO group were sacrificed at each time point). The kidney tissue was collected from the obstructed side for western blotting, real-time PCR, and pathological analysis.

\textbf{Kidney tissue pathological examination}

The kidney tissue was fixed in 10% neutral formaldehyde, dehydrated with ethanol, embedded in paraffin, and cut into 2 µm sections. Morphological changes in the kidney tissue were observed by periodic acid Schiff (PAS) and Masson's trichrome staining. After staining with Masson's trichrome, 10 fields of vision were selected under a light microscope (400×). The area of each field of vision and area of green collagen fibers were measured using the Image-Pro Plus software. The relative area of collagen deposition was calculated was follows: area of green collagen fiber/area of field of vision × 100%.

\textbf{Statistical analysis}

The data were analyzed using the SPSS 17.0 software, and the results were expressed as mean ± standard error of mean (SEM). The differences among the experimental groups were analyzed using the one-way analysis of variance (ANOVA) with completely random design. P < 0.05 was considered statistically significant.

\textbf{Results}

\textbf{Inhibitory effect of TGF-\textbeta1 on miR-133b}

The real-time PCR results of miR-133b expression analysis showed that the stimulation of HK2 cells with TGF-\textbeta1 at 0, 6, 8, and 10 ng/mL for 48 h resulted in a gradual decrease in the expression of miR-133b in a
TGF-β1 concentration-dependent manner. However, no significant difference was observed between the 8 and 10 ng/mL treatment groups (Figure.1A). The expression of miR-133b after stimulation of HK2 cells with TGF-β1 at 8 ng/mL for 0, 24, 48, and 72 h gradually decreased with an increase in the stimulation time; no significant difference was observed between the 48 and 72 h treatment groups (Figure.1B).

**Effect of miR-133b on the morphology of TGF-β1-stimulated HK2 cells**

Morphological changes in cells were observed under an inverted microscope. HK2 cells had a round or an oval shape and were arranged in a cobblestone pavement-like pattern. After stimulation with 8 ng/mL TGF-β1 for 48 h, the cells became slender and fusiform, and their intercellular space significantly widened. Most transfected miR-133b cells could still maintain the morphology of epithelial cells, while the NC-miR-transfected cells had morphological changes similar to those observed for HK2 cells (Fig. 2).

**miR-133b inhibited the EMT of HK2 cells induced by TGF-β1**

Normal HK2 cells or HK2 cells transfected with miR-133b mimic/NC-miR for 24 h were stimulated with 8 ng/mL TGF-β1 for 48 h. The results of real-time PCR and western blotting showed that miR-133b significantly inhibited the downregulated E-cadherin mRNA and protein expression as well as the upregulated mRNA and protein expression of α-SMA, fibronectin, Col3A1, and CTGF induced by TGF-β1 (Fig. 4A and 4B).

Immunofluorescence results showed that the fluorescence intensity of α-SMA increased and that of E-cadherin decreased in the cells treated with TGF-β1 as compared with that in control cells. In comparison with the TGF-β1 stimulation group, the cells treated with TGF-β1 and miR-133b showed a decrease in the fluorescence intensity of α-SMA and an increase in the intensity of E-cadherin. Further, we failed to notice any significant change in the fluorescence intensity of α-SMA and E-cadherin between the cells treated with TGF-β1 and NC-miR-133b and those treated with TGF-β1 alone (Table 1; Fig. 4C).
Table 1
Comparison of the fluorescence intensity of α-SMA and E-cadherin in HK2 cells under different stimulation conditions.

| Group                | Number of experiments | Average fluorescence intensity of cells |
|----------------------|-----------------------|----------------------------------------|
|                      |                       | α-SMA       | E-cadherin  |
| Control              | 3                     | 0.021 ± 0.003 | 0.175 ± 0.028 |
| TGF-β1               | 3                     | 0.103 ± 0.043* | 0.013 ± 0.001* |
| TGF-β1 + miR-133b    | 3                     | 0.041 ± 0.005## | 0.067 ± 0.009## |
| TGF-β1 + NC-miR      | 3                     | 0.092 ± 0.004* | 0.009 ± 0.001* |

Legend: The experiment was performed in triplicates, and five fields of vision were selected for each group. The fluorescence intensity of cells was detected under a confocal microscope (100×); *: P < 0.05 versus control group; #: P < 0.05 versus TGF-β1 group.

CTGF is a direct target gene of miR-133b

We used three different miRNA target gene prediction software (TargetScan, PiTar, and miRbase) and found CTGF as a potential target of miR-133b. The target site was located at 1027 to 1033 bp of CTGF 3'-UTR. The results of DAVID bioinformatic prediction suggested that CTGF is involved in TGF-β fibrosis and other signaling pathways.

After the transient transfection of miR-133b mimic into HK2 cells for 48 h, we performed real-time PCR and western blotting and found a significant increase in miR-133b expression (increased by about 21,566 times) (Fig. 3A), confirming the successful transfection of miR-133b. The expression of CTGF mRNA and protein significantly decreased following miR-133b transfection but showed no changes in the group transfected with an unrelated sequence (Fig. 3B and 3C).

The cells were transfected with CTGF for 48 h, and then collected and analyzed by western blotting. In comparison with the HK2 cells transfected with GAPDH, those transfected with CTGF showed a significant upregulation in the expression of CTGF protein, confirming the successful transfection of CTGF (Fig. 5A). We stimulated HK2 cells with TGF-β1 at 8 ng/mL for 48 h and performed western blotting. We found that the miR-133b overexpression group showed a significant downregulation in the expression of CTGF, α-SMA, fibronectin, and Col3A1 and a significant upregulation in the expression of E-cadherin as compared with the control group. The cells overexpressing miR-133b and CTGF showed a significant increase in CTGF expression. CTGF could revert the inhibition of α-SMA, fibronectin, and Col3A1 expression and the increase in E-cadherin expression mediated by miR-133b overexpression. Furthermore, the group overexpressing miR-133b and CTGF showed significantly higher CTGF and α-SMA levels and significantly lower E-cadherin levels than the control group. The group overexpressing miR-133b and GAPDH showed no significant difference in the expression of various proteins as compared with the miR-133b overexpression group (Fig. 5B).
The results of dual-luciferase reporter assay showed that miR-133b mimic significantly decreased the luciferase activity of miRGLO-Wt-CTGF but had no significant effect on the luciferase activity of miRGLO-Mut-CTGF (Fig. 3D).

**miR-133b inhibited renal interstitial fibrosis and reduced renal function loss in old UUO mice**

The miR-133b or NC-miRNA transfection complex was administered to UUO + miR-133b or UUO + NC-miR mice by intravenous injection, respectively, and the expression of miR-133b was detected by real-time PCR. At 7 and 14 days after UUO establishment, the expression of miR-133b was significantly higher in the UUO + miR-133b group than in the sham and UUO + NC-miR groups, confirming the successful transfection of miRNA-133b. The expression of miRNA-133b in the UUO + NC-miR group was significantly lower than that in the sham group (Fig. 6A).

PAS and Masson staining techniques were used to observe the morphology of the kidney tissue. In comparison with the sham group, the UUO + NC-miR group showed interstitial edema, tubular dilatation, interstitial inflammatory cell infiltration, and a slight increase in collagen level at 7 days after UUO operation on the ureter ligation side of the kidney. At 14 days after UUO operation, the interstitial cell population increased and collagen expression was significantly upregulated. In comparison with the UUO + NC-miR group, the UUO + miR-133b group showed a significant decrease in the level of collagen in the renal interstitium at 7 and 14 days after UUO operation (Fig. 6B).

We observed the relative area of collagen deposition in the kidney tissue and found it to be significantly higher in all UUO model animals than in the sham group. The value observed for UUO + miR-133b group was significantly lower than that reported for the UUO + NC-miR group at 7 and 14 days after UUO operation (Table 2).
Table 2
Comparison of the relative area of collagen deposition in the kidney tissue of animals from each group.

| Group              | n  | Relative area of collagen deposition (%) |
|--------------------|----|-----------------------------------------|
| Sham-7d            | 4  | 4.10 ± 0.3                               |
| Sham-14d           | 4  | 4.09 ± 0.3                               |
| UUO-7d + NC-miR    | 5  | 38.17 ± 5.67*                            |
| UUO-14d + NC-miR   | 5  | 82.52 ± 7.10*                            |
| UUO-7d + miR-133b  | 5  | 17.83 ± 4.16*#                           |
| UUO-14d + miR-133b | 5  | 40.34 ± 6.18*#                           |

Legend: After Masson trichrome staining, 10 fields for each animal were observed under a light microscope (400×), and the area of each field as well as the area of green collagen fiber were measured by Image-Pro Plus software. The relative area of collagen deposition was calculated as follows: Area of green collagen fiber/Area of field of vision × 100%. *: P < 0.01 versus sham group; #: P < 0.01 versus UUO + NC-miR group.

The results of real-time PCR and western blotting showed that the mRNA and protein levels of CTGF, fibronectin, Col3A1, and α-SMA in the UUO + NC-miR group were significantly higher and those of E-cadherin were significantly lower than the values reported in the sham group. Further, the changes were more significant at 14 days as compared with those at 7 days after UUO operation. At both 7 and 14 days after UUO operation, the mRNA and protein levels of CTGF, fibronectin, Col3A1, and α-SMA significantly decreased and those of E-cadherin significantly increased in the UUO + miR-133b group as compared with the values observed for the UUO + NC-miR group. However, the mRNA and protein levels of CTGF, fibronectin, Col3A1, and α-SMA in the UUO + miR-133b group were still significantly higher and those of E-cadherin were significantly lower than the values for the sham group.

The results of renal function test showed no significant difference in the blood urea nitrogen (BUN) and serum creatinine (sCr) levels between the three groups at day 0. At day 7 following UUO operation, BUN and sCr levels significantly increased in UUO mice as compared with those in sham mice. The BUN level in the UUO + miR-133b group was significantly lower than that in the UUO + NC-miR group. At 14 days after UUO operation, BUN and sCr levels in UUO mice were significantly higher than those in the sham mice and those in the UUO + miR-133b group were significantly lower than the values reported for the UUO + NC-miR group (Table 3).
### Table 3
Comparison of the renal functions among different groups of animals.

| Groups        | 0 days | 7 days | 14 days |
|---------------|--------|--------|---------|
|               | n      | BUN (mmol/L) | sCr (µmol/L) | n | BUN (mmol/L) | sCr (µmol/L) | n | BUN (mmol/L) | sCr (µmol/L) |
| Sham         | 8      | 6.9 ± 1.0   | 17.4 ± 0.7  | 4 | 6.7 ± 1.0   | 17.2 ± 0.7  | 4 | 7.0 ± 1.4   | 17.3 ± 1.0  |
| UUO + NC-miR | 10     | 6.9 ± 1.0   | 17.7 ± 2.1  | 5 | 13.8 ± 1.7* | 22.5 ± 1.2* | 5 | 18.3 ± 1.5* | 28.4 ± 0.7* |
| UUO + miR-133b | 10    | 6.7 ± 0.6   | 17.1 ± 1.9  | 5 | 10.3 ± 1.1*#| 20.5 ± 1.7* | 5 | 14.1 ± 1.3*#| 23.2 ± 1.5*#|

Legend: BUN: blood urea nitrogen; sCr: serum creatinine; *: P < 0.05 versus sham group; #: P < 0.05 versus UUO + NC-miR group.

### Discussion

Tubulointerstitial fibrosis is a chronic and progressive process affecting the kidney tissue during aging as well as in chronic kidney disease regardless of the underlying cause [12]. Kidney fibrosis is characterized by the EMT of tubular epithelial cells [13] that contributes to both the destruction of the tubular epithelial compartment and accumulation of interstitial fibroblasts [14]. TGF-β signaling is thought to play a predominant role in this process [15]. CTGF is a direct downstream early response factor of TGF-β, also known to potentiate TGF-β signaling by directly binding TGF-β through its CR domain [16].

The miRNA miR-133 was first experimentally characterized in mice, and its homologs were identified in several other species, including invertebrates such as the fruit fly *Drosophila melanogaster* [17]. In the human genome, miR-133 genes include miR-133a-1, miR-133a-2, and miR-133b located on chromosomes 18, 20, and 6, respectively [18]. miR-133 is necessary for the proper development and function of skeletal and cardiac muscles, and its aberrant expression has been linked to many diseases of the skeletal and cardiac muscles. It is identified as a key factor in cancer development [17–20] and is also known to alleviate cardiac fibrosis in many animal models [8–10]. Lentiviral transfection of miR-133 was found to reduce renal interstitial fibrosis in old UUO mice [7] and renal fibrosis in diabetic rats [21]. In the present study, we induced the high expression of miR-133b in the kidney tissue through an intravenous injection of an miR-133b transfection complex. It was found that miR-133 downregulated the mRNA and protein expression of CTGF, fibronectin, Col3A1, and α-SMA and upregulated the mRNA and protein levels of E-cadherin, thereby significantly alleviating renal interstitial fibrosis and reducing the loss of renal function in old UUO mice. We, thus, reconfirmed the effect of miR-133 on geriatric renal interstitial fibrosis.

The effect of miR-133 on the EMT induced by TGF-β1 is controversial. miR-133 was previously shown to inhibit the EMT of HK2 cells induced by TGF-β1 [6, 7]. TGF-β1 downregulated the expression of miR-133a/b in the bladder smooth muscle epithelial cells in a concentration-dependent manner, and
transfection with miR-133 mimics resulted in the attenuation of the TGF-β1-induced α-SMA, extracellular matrix subtype, and fibrotic growth factor expression [11]. Treatment of primary murine and human hepatic stellate cells with TGF-β1 resulted in a significant downregulation in the expression of miR-133a. On the other hand, the overexpression of miR-133a in primary murine hepatic stellate cells was shown to decrease the expression of collagen [22]. However, miR-133b was found to be overexpressed in TGF-β1-treated HK-2 cells, and miR-133b inhibition attenuated the TGF-β1-induced EMT of HK-2 cells [21]. Our experiments confirmed the TGF-β1-mediated downregulation of miR-133 expression in a concentration- and time-dependent manner, and showed that the overexpression of miR-133b significantly inhibited the downregulation in the mRNA and protein expression of E-cadherin as well as the upregulation in the mRNA and protein levels of α-SMA, fibronectin, Col3A1, and CTGF induced by TGF-β1. The inhibitory effect of miR-133 on the EMT of HK2 cells induced by TGF-β1 was further clarified.

Only a few studies have evaluated the effect of target genes of miR-133 on the alleviation of tissue fibrosis. Only a single renal fibrosis study confirmed Sirtuin-1 as a target of miR-133b in HK-2 cells and showed that the inhibition of miR-133b expression resulted in the attenuation of the TGF-β1-induced EMT and renal fibrosis through the upregulation of Sirtuin-1 expression [21]. Further, CTGF was found as a direct target of miR-133 during the EMT of cardiomyocytes [9] and bladder smooth muscle epithelial cells [11]. The overexpression of miR-133 significantly downregulated the mRNA and protein levels of CTGF, and CTGF overexpression could reverse the inhibitory effect of miR-133b on the TGF-β1-induced EMT of HK2 cells. The results of dual-luciferase reporter assay also confirmed that CTGF is a direct target of miR-133. Therefore, our study confirmed for the first time that CTGF is a target of miR-133 and is involved in ameliorating renal fibrosis.

CTGF not only potentiates TGF-β signaling by directly binding TGF-β1 but also modifies various growth factors and cytokines. Each domain of CTGF can bind to multiple ligands, including insulin-like growth factor-1, fibronectin, bone morphogenetic factors, α5β3 integrin, low-density lipoprotein receptor-related protein 1, vascular endothelial growth factor (VEGF), Wnt, integrins, heparan sulfate proteoglycan, receptor-related proteins, and epidermal growth factor receptor [22]. These cytokines can participate in kidney aging and renal fibrosis through various signaling pathways. Therefore, miR-133 may play different biological roles by downregulating the expression of CTGF and influencing the processes of kidney aging and renal fibrosis. These effects need to be confirmed through future studies.

Conclusions

We showed that CTGF is a target gene of miR-133 involved in ameliorating renal fibrosis and clarified the miR-133b-mediated inhibition of the EMT of HK2 cells induced by TGF-β1 that resulted in the alleviation of renal interstitial fibrosis in old UUO mice. These observations serve as a basic research evidence for the development of new drugs based on miR-133 to ameliorate kidney aging and renal interstitial fibrosis.

Declarations
Acknowledgements

Not applicable.

Authors’ contributions

Xuefeng Sun participated in conceiving, designing and coordinating this study. Animal experiments were conducted by Dan Cao, Yingjie Zhang, Qi Huang and Zhong Yin. Cell experiments were performed by Dan Cao, Yuan Wang and Yinpeng Zhang. Statistical analyses of the data were completed by Dan Cao, Yuan Wang and Xuefeng Sun. The manuscript was written and revised by Dan Cao, Yuan Wang, Xuefeng Sun, Guangyan Cai and Xiangmei Chen. All authors read and approved the final manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

Animal welfare was ensured, and experimental procedures were all carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

MSC: miR: MicroRNA; MSCs: Mesenchymal stromal cell; EVs: Extracellular vesicles; MSC-EVs: MSC derived extracellular vesicles; TGF-β1: Transforming growth factorβ1; EMT: Epithelial-Mesenchymal Transition; HK2: Human renal proximal tubular epithelial; α-SMA: alpha-Smooth Muscle Actin; COL3A1: COL3A1;
alpha 1 chain of type III collagen; UUO: Unilateral ureteral obstruction; CTGF: Connective tissue growth factor; NC-miR: negative control microRNA; FITC: Fluorescein isothiocyanate; PAS staining: periodic acid-schiff staining; BUN: Blood urea nitrogen; sCr: Serum creatinine;

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Figures

![Figure 1](image-url)

Figure 1
Expression of miR-133b in HK2 cells stimulated with different concentrations of TGF-β1 for different time points. Legend: The experiment was performed in triplicates, and the expression of miR-133b was detected by real-time PCR. A: The expression of miR-133b in HK2 cells stimulated with different concentrations of TGF-β1. a: P<0.05 versus 0 ng/mL TGF-β1 group; b: P<0.05 versus 6 ng/mL TGF-β1 group. B: The expression of miR-133b in HK2 cells stimulated with TGF-β1 at 8 ng/mL for different time points. c: P<0.05 versus 0 h group; d: P<0.05 versus 24 h group.

Figure 2

Morphological changes in HK2 cells following different stimulation conditions. Legend: The morphological changes in cells were observed under an inverted microscope (100×). Control: HK2 cells were cultured for 48 h without stimulation; TGF-β1: HK2 cells were stimulated with 8 ng/mL TGF-β1 for
48 h; TGF-β1+miR-133b: HK2 cells transfected with miR-133b were stimulated with TGF-β1 at 8 ng/mL for 48 h; TGF-β1+NC-miR: HK2 cells transfected with NC-miR were stimulated with TGF-β1 at 8 ng/mL for 48 h.

Figure 3

CTGF is a direct target gene of miR-133b. Legend: Transient transfection of miR-133b mimic into HK2 cells for 48 h. A: miR-133b expression was detected by real-time PCR. B: CTGF expression was detected
miR-133b inhibited the EMT of HK2 cells induced by TGF-β1. Legend: Normal HK2 cells or HK2 cells transfected with miR-133b mimic/NC-miR for 24 h were stimulated with 8 ng/mL TGF-β1 for 48 h. A: Real-time PCR results. B: Western blotting results. C: Cell immunofluorescence assay results. a: P<0.05 versus control group; b: P<0.05 versus TGF-β1 group.
Figure 5

miR-133b inhibited the EMT of HK2 cells induced by TGF-β1. Legend: A: Western blotting results after transfection of HK2 cells with CTGF for 48 h. B: Western blot analysis results of HK2 cells stimulated with TGF-β1 at 8 ng/mL for 48 h. a: P<0.05 versus control group; b: P<0.05 versus miR-133b group.
Figure 6

miR-133b inhibited renal interstitial fibrosis in old UUO mice. Legend: A UUO mouse model was administered with an intravenous injection of miR-133b or NC-miRNA transfection complex; A: Real-time PCR for miRNA-133b expression analysis. B: Masson and PAS staining of renal tissue and observation under a light microscope (100×). a: P<0.05 versus sham group; b: P<0.05 versus UUO+NC-miR group.