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Original article

# CTRP6 inhibits cell proliferation and ECM expression in rat mesangial cells cultured under TGF-β1

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ABSTRACT

C1q/tumor necrosis factor-related protein 6 (CTRP6), a member of CTRPs family, was involved in fibrosis. However, the biological function of CTRP6 in renal fibrosis remains elusive. This study aimed to examine the role of CTRP6 in renal fibrosis and explore the possible mechanism. Our results demonstrated that the expression of CTRP6 was significantly downregulated in renal fibrotic tissues and TGF-β1-treated NRK-49F cells. In addition, overexpression of CTRP6 inhibited the proliferation, migration, and ECM expression in TGF-β1-treated NRK-49F cells. Furthermore, overexpression of CTRP6 attenuated TGF-β-induced phosphorylation of ERK1/2 in NRK-49F cells. The ERK1/2 inhibitor U0126 enhanced the inhibitory effects of CTRP6 overexpression on cell proliferation, migration and ECM expression in TGF-β1-stimulated NRK-49F cells. In conclusion, we have demonstrated that CTRP6 suppressed ECM expression in renal fibroblasts induced by TGF-β1 through the ERK signaling pathway. Therefore, CTRP6 may be a potential therapeutic target for the treatment of renal fibrosis.

## 1. Introduction

Renal fibrosis is a typical pathological feature during the progression of chronic kidney disease. It is characterized by the overproduction and accumulation of extracellular matrix (ECM) proteins [1]. Although various treatment methods widely apply for renal fibrosis [2–4], the pathogenesis and therapeutic interventions of renal fibrosis are not well established. Transforming growth factor (TGF)-β1 is a key fibrogenetic cytokine involved in renal fibrosis. It was reported that TGF-β1 stimulates ECM production, leading to progressive renal fibrosis [5]. Thus, suppression of TGF-β1-induced ECM accumulation is the main approach for the treatment of renal fibrosis.

C1q/TNF-related proteins (CTRPs), a new highly conserved family of adiponectin paralogs, were reported to be implicated in a variety of processes including metabolism, immunity, inflammation, cell differentiation, and organogenesis [6–8]. C1q/tumor necrosis factor-related protein 6 (CTRP6) is a member of CTRPs family and contains four domains including signal peptide, short N-terminal variable region, collagen domain and C-terminal C1q domain [9]. It plays critical roles in cardiovascular diseases, inflammatory reaction and adipogenesis [10,11]. In addition, several studies showed that CTRP6 possesses antifibrotic effect [12]. For example, Lei et al. reported that CTRP6 improved cardiac function, attenuated cardiac hypertrophy, alleviated cardiac fibrosis, and inhibited myofibroblast differentiation post-myocardial infarction [13]. However, the biological function of CTRP6 in renal fibrosis remains elusive. This study aimed to examine the role of CTRP6 in renal fibrosis and explore the possible mechanism. These results demonstrated that CTRP6 suppressed ECM expression in renal fibroblasts induced by TGF-β1 through the ERK signaling pathway.

## 2. Materials and methods

### 2.1. Tissue sample collection

Renal biopsy samples were obtained from six healthy individuals and six patients with renal fibrosis at the Department of Nephrology, Huaihe Hospital of Henan University (China). Samples were snap frozen in liquid nitrogen and stored at −80 °C. This study was approved by Ethics Committee of Huaihe Hospital of Henan University. Informed consent was obtained from all individual subjects for all procedures.

### 2.2. Cell culture

The rat kidney fibroblast cell line (NRK-49F) was obtained from the American Type Cell Collection (ATCC, Manassas, VA, USA). NRK-49F cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Sigma,

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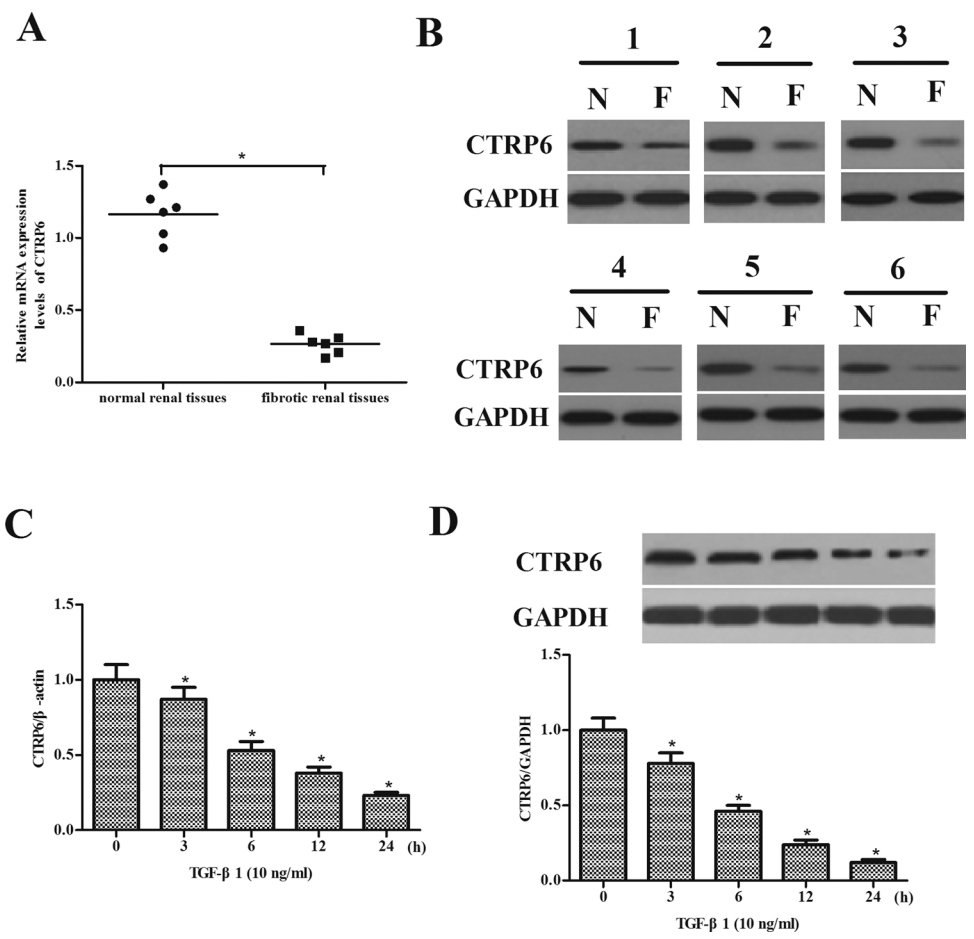


**Fig. 1.** CTRP6 was downregulated in renal fibrotic tissues and TGF-β1-treated NRK-49F cells. A, The mRNA expression levels of CTRP6 were detected in human fibrotic renal tissues and normal renal tissues, $*p < 0.05$ vs the human normal renal tissues. B, The protein expression levels of CTRP6 were detected in human fibrotic renal tissues and normal renal tissues, $*p < 0.05$ vs the human normal liver tissues. N, normal renal tissues; F, fibrotic renal tissues. C and D, NRK-49F cells were treated with TGF-β1 (10 ng/ml) for different hours. The mRNA and protein levels of CTRP6 were determined using qRT-PCR and western blot assays. Data were expressed as means ± SD. $*p < 0.05$ vs. the control group.

St. Louis, MO, USA) in a $CO_2$ incubator with a humidified atmosphere of 5% $CO_2$ at 37 °C.

### 2.3. Plasmid constructs and cell transfection

The full length cDNA-encoding sequence of CTRP6 was sub-cloned into pcDNA3.1 vectors according to standard protocols, and pcDNA3.1 empty vector was used as a negative control. NRK-49F cells at a density of $1 \times 10^5$ cells/well were seeded into 96-well microplates, grown for 24 h to reach 80% confluence, and transfected with pcDNA3.1-CTRP6 or pcDNA3.1 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

### 2.4. RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from tissues or cells using Trizol reagent (Takara Biotechnology, Dalian, China). Approximately 4 μg total RNA for each sample were reverse transcribed into first strand cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Invitrogen). The levels of gene mRNA transcripts were analyzed by using specific primers and SYBR Green I reagent and the RT-PCR kit, according to the manufacturer's instructions, using the Bio-Rad iQ5 Quantitative PCR System (Takara, Dalian, China). The specific primers for CTRP6 were forward, 5′-CAGATGGTGGAAACTTTTAGCC-3′ and reverse, 5′-AACTTCCTTAGC ACCTGGATCA-3′; and β-actin forward, 5′-GAGGCA CTCTTCCAG CCTTC-3′ and reverse, 5′-GGATGTCCACGTCACACTTC-3′. The steps used for RT-qPCR were as follows: 94° C for 2 min for initial denaturation; 94° C for 10 s, 59° C for 25 s, and 72° C for 30 s; 2 s for plate reading for 35 cycles; and a melt curve from 65 to 95° C. The relative expression was calculated with the $2^{-\Delta\Delta CT}$ equation. All of the reactions were run in triplicate.

### 2.5. Western blot analysis

Cells or tissues were homogenized and lysed using RIPA lysis buffer (Beyotime, Nantong, China). 30 μg of protein per lane was separated by 12% SDS-PAGE and transferred on polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membranes were blocked with 10% defatted milk in TBST buffer at room temperature for 1 h, and then incubated with primary antibodies (anti-CTRP6, anti-collagen I, anti-α-SMA, anti-*p*-ERK1/2, anti-ERK1/2 and anti-GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. After incubated with appropriate HRP-conjugate secondary antibodies, the signals were determined using an enhanced chemiluminescence (Gibco, Rockville, MD).

### 2.6. Cell proliferation assay

Cell proliferation was evaluated using the cell counting kit-8 assay (CCK-8; Dojindo, Kumamoto, Japan). In brief, NRK-49F cells were transfected with pcDNA3.1-CTRP6 or pcDNA3.1 for 24 h, respectively, and then treated with TGF-β1 (10 ng/ml) for 24 h. Next, 10 μl of reagent from CCK-8 (Dojindo, Kumamoto, Japan) was added into each well, and the plate was incubated for 1 h at 37 °C. The absorbance was read at 490 nm using an enzyme linked immunosorbent assay plate reader (Olympus, Tokyo, Japan). Each cell proliferation assay was performed in quadruplicate and repeated three times.