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WISP3 prevents fibroblast–myofibroblast transdifferentiation in NRK-49F cells

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ABSTRACT

CCN family, a group of six extracellular matrix-associated proteins, plays an important role in fibrosis. WISP3 has addressed as a pro-fibrotic molecule in the development of human lung fibrosis. However, whether WISP3 involved in the activation and proliferation of renal fibroblast, and ultimately inhibited fibroblast–myofibroblast transdifferentiation remained unknown. Herein, we found that down-regulated WISP3 was involved in the fibrogenesis of rat renal NRK-49F cells induced by transforming growth (TGF-β1), which was further confirmed in a rat renal fibrosis induced by unilateral ureteral obstruction (UUO). In the present study, we aimed to investigate the roles of WISP3 in NRK-49F fibroblast–myofibroblast transdifferentiation, and the underlying mechanism. Results showed that after TGF-β1 treatment, significant increased cell proliferation, and up-regulated expressions of TGF-β1, connective tissue growth factor (CTGF), α-smooth muscle actin (α-SMA), vimentin, as well as increased concentrations of collagen types I (COL I), collagen types III (COL III) and hydroxyproline in cell culture supernatant were observed, demonstrating a successful establishment of fibroblast–myofibroblast transdifferentiation of NRK-49F cells. Besides, siRNA-WISP3 remarkably promoted the fibrogenesis of NRK-49F cells with or without TGF-β1 treatment, and increased mRNA levels of Axin, demonstrating that activating WNT signaling pathway was the underlying mechanism. However, lentivirus-mediated WISP3 overexpression exerted an opposite effect, protecting NRK-49F cells from transdifferentiation, and decreasing mRNA levels of Axin. In conclusion, the WISP3 played an anti-fibrotic role in NRK-49F cells, and WNT signaling pathway was the potential mechanism. WISP3 was an anti-fibrotic factor in fibroblast–myofibroblast transdifferentiation, and may be used as a possible target for prevention and treatment of human renal fibrosis.

1. Introduction

Renal fibrosis, also known as tubule interstitial fibrosis, is characterized with glomerular sclerosis and accumulation of extracellular matrix proteins (ECM) in tubule interstitium [1]. In addition to known as a common consequence of a variety of chronic kidney diseases (CKD), renal fibrosis is also a major risk factor in the development of CKD, which will inevitably lead to chronic renal failure, a devastating disorder that requires dialysis or kidney transplantation, and thus creates a huge financial burden on families and our medical institutions. So far, renal progressive fibrosis still remains unsolved challenges for our nephrologists.

The fibrotic process is enormously complicated, generally divided four overlapping phases: (a) myofibroblasts/activated fibroblasts; (b) fibrogenic signaling phase, generating various fibrosis-promoting factors; (c) matrix proteins accumulating; (d) renal destruction, characterized with a continuous reduction in glomerular filtration [2,3]. Because of multiple cellular events, molecular mediators, and even the interacting between them, fibrotic process has not been understood completely and still remains a challenge for us. As far as we know, among those factors, transforming growth factor-β (TGF-β) plays a central role. TGF-β1 mediates some key tubular pathological events, including the regulation of fibroblast proliferation, the production of epithelial to mesenchymal transition (EMT), ECM accumulation and epithelial cell death [4,5]. In animals, overproduction of TGF-β1 by intravenous injection or gene pathways contributes to rapid renal fibrosis [6,7]. Treating fibroblasts isolated from humans, rats, mice and pigs with TGF-β1 can model this disease for identifying endogenous or exogenous anti-fibrotic factors, or even for further putting insight into the mechanisms involved, and thus providing us with unprecedented possibilities for therapeutic interventions of renal fibrosis.

Huge body of evidence suggests that the members of CCN family (a group of six ECM-associated proteins) play an important role in renal fibrosis process. CCN family includes CYR61 (cysteine-rich 61);

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connective tissue growth factor (CTGF), nephroblastoma overexpressed gen (NOV) and Wnt1-inducible signaling proteins (WISP1, WISP2 and WISP3). CCN family proteins have one of characteristic structural domains that contain carboxy terminal cystine knots (CTs), for example cerberus, sclerostin (SOST), noggin and the superfamily of TGF-β [8,9]. CYR61, NOV and WISP2 have been reported as anti-fibrotic factors [10–12], while CTGF and WISP1 have been served as profibrotic molecules [13,14]. WISP3 stimulates the proliferation of lung fibroblasts, and is recognized as a pro-fibrotic mediator in lung fibrosis [15]. However, whether WISP3 involved in renal fibrosis remains extremely poor. Therefore, to explore the roles of WISP3 in renal fibrogenesis, NRK-49F cells, a type of renal interstitial fibroblasts, were stimulated by TGF-β1 to establish a fibroblast–myofibroblast transdifferentiation cell model. Besides, siRNA-WISP3 and lentivirus-mediated WISP3-overexpression were involved. The study here may provide a new possibility targeted WISP3 to block human kidney fibrosis.

2. Materials and methods

2.1. Cell culture and treatment

NRK-49F cell line is a type of rat renal interstitial fibroblasts, and came from Shanghai Institute for Biological Science. NRK-49F cells were maintained at 37 °C under 5% CO2 in DMEM (high-glucose medium, HyClone, Int., USA) adding 1% penicillin and 10% fetal bovine serum. Adherent cells were treated with trypan blue staining, the number of cells was observed under a microscopy. The cell survival rate was 95% or more. To study the effect of TGF-β1 on WISP3 expression in NRK-49F cells, cells were treated with recombinant human TGF-β1 (100-21, Peprotech) at different concentrations (2, 5 and 10 ng/mL), and then cultured as described above. To study the effect of WISP3 protein on NRK-49F cell transdifferentiation, cells were treated with TGF-β1 (10 ng/mL) and recombinant human WISP3 (120-20, Peprotech) at a concentration of 0.5 ug/mL, and then cultured as mentioned above.

2.2. Production and transfection of WISP3 overexpression vectors

The lentiviral vector system consists of three main components before packaging, including the pLVX-Puro vector (Clontech, Laboratories, Inc.), psPAX2 and pMD2G vector (Addgen). The pLVX-Puro vector was used for encoding human WISP3 gene (NCBI NM_001170483.1). The primers used were 5′ -GCGAATTCATGCACAG ACTTCTCTTTTGCACTC-3′ (WISP3-forward) and 5′-CGGGATCCCTATA GGATCCTGAGCTCAGAAAAT-3′ (WISP3-reverse), with EcoR1 and BamH I sites, respectively. The accurate insertion of the WISP3 cDNA was confirmed by Shanghai Majorbio Bio-Pharm Technology Co., Ltd. pLVX-Puro-WISP3, psPAX2, pMD2G were extracted with Endo-free Plasmid Mini Kit I (OMGEA, E.Z.NA®), and then cotransfected into 293T cells (ATCC, Shanghai, China) using Lipofectamine™ 2000 according to manufacturer's protocol (Invitrogen Co., Carlsbad, CA). After 4–6 h, the medium was exchanged completely. High-titer recombinant lentiviruses with WISP3 were obtained 48 h and 72 h. Cells infected with WISP3 overexpression vector were considered as WISP3 overexpression group, meanwhile, cells infected with the corresponding empty vector without WISP3 expression was considered as vector control group.

2.3. Production and transfection of WISP3 silencing vectors

The lentivirus vector system consists of three components: PLKO.1, psPAX2 and pMD2G (Addgen). The PLKO.1 vector contains an U6 promoter controlled expression of short hairpin RNAs (shRNAs). A 20–23 nt fragment within the WISP3 cDNA was chosen as the target for our siRNA (siRNA-WISP3): 5′- ACACAAGAGTGACTATTCT-3′ at position 1158–1180 corresponding to WISP3 mRNA (GenBank accession No. NM_001170483.1). The designed shRNA was synthesized and inserted to PLKO.1 expression vector (pLKO.1-shWISP3). Accurate insertion of designed shRNA was confirmed by Shanghai Majorbio Bio-Pharm Technology Co., Ltd. The lentivirus vector system was packaged, then cotransfected into 293T cells (ATCC) as the same method mentioned above. The scrambled shRNA plasmid, serving as a negative control, was constructed in the same manner. Cells infected with WISP3-siRNA-pLKO.1 were considered as siRNA-WISP3 groups, meanwhile, cells infected with a scrambled shRNA plasmid were considered as the corresponding control (siRNA-NC).

2.4. Rat renal interstitial fibrosis

Unilateral ureteral obstruction (UUO)-induced rat renal interstitial fibrosis was constructed according to the reported method [16], and were directly available in our current study. Six rats weighting 200–250 g were randomly divided into two groups (n = 3): normal and renal fibrosis groups. Rats in renal fibrosis group were subjected to UUO, and three kidneys were harvested at eight weeks after surgery. Healthy rats without UUO treatment were considered as the corresponding normal group. Herein, we declared that the experimental procedures and the animal use and care protocols were approved by the local committee on ethical use of animals of Huashan Hospital affiliated to Fudan University Hospital.

2.5. Immunohistochemistry (IHC) assay

Meanwhile, IHC analysis was performed in normal and fibrotic kidneys to observe the expression pattern of WISP3. Formalin-fixed paraffin-embedded renal tissue sections (4 mm thick) were processed for IHC staining as previously described. The antibodies used in this study were as following: rabbit polyclonal anti-WISP3 (ab224720, Abcam, Cambridge, MA, USA) and upervision TM mouse/rabbit-HRP-broad spectrum detection system (Long Island Biotech, China). DAB-containing substrate kit (FL-6001, Long Island Biotech, China) was used to visualize interstitial cells. 4,6-diamidino-2-phenylindole (DAPI) was used to stained nuclei. WISP3-positive areas were calculated using i-solution software and determined in 10 fields/kidney (n = 3 per group).

2.6. Cell proliferation analysis

The numbers of viable cells were detected by colorimetric cell counting kit-8 (CCK8; SAB). NRK-49F cells suspension (30,000 cells/well; 100 μL) was seeded into 96-well plates. After TGF-β1 treatment or viral transduction, cells were cultured as described above. At 0 h, 24 h, 48 h and 72 h, CCK-8 solution (10 μL) and serum-free medium (90 μL) were added to each well, then incubated at 37 °C under 5% CO_2 for 1 h. The absorbance at 450 nm was read using a microplate reader. For each cell line, cell survival rates were detected at three time points of the cell growth curve through the log phase of growth.

2.7. Enzyme-linked immunosorbent assay (ELISA) assay

Levels of Collagen type I (COL I) and Collagen type III (COL III) in the supernatants were assessed using ELISA method, following the manufacturer's protocol (eBioscience, San Diego, CA).

2.8. Hydroxyprolineassay

Levels of hydroxyproline in the supernatants were quantified using the commercially available bioassays (www.njjcbio.com).

2.9. Real-time PCR

Total RNA was isolated using a guanidiniumisothiocyanate/chloroform based technique (Trizol; Invitrogen, San Diego, CA, USA).