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Up-regulation of microRNA-93 inhibits TGF-β1-induced EMT and renal fibrogenesis by down-regulation of Orai1

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ABSTRACT

TGF-β1-induced excessive deposition of ECM and EMT process of tubular epithelial cells play critical roles in the development and progression of fibrosis in diabetic nephropathy (DN). Orai1 has been demonstrated to be involved in TGF-β1-induced EMT via TGF-β/Smad3 pathway. We are aimed to explore the effects of miR-93 on TGF-β1-induced EMT process in HK2 cells. In this study, our data showed that miR-93 was dramatically decreased in renal tissues of patients with DN and TGF-β1-stimulated HK2 cells. Moreover, the decreased level of miR-93 was closely associated with the increased expression of Orai1. Overexpression of miR-93 decreased Orai1 expression, and then suppressed TGF-β1-mediated EMT and fibrogenesis. Next, we predicted that the Orai1 was a potential target gene of miR-93, and demonstrated that miR-93 could directly target Orai1. SiRNA targeting Orai1 was sufficient to suppress TGF-β1-induced EMT and fibrogenesis in HK2 cells. Furthermore, Overexpression of Orai1 partially reversed the protective effect of miR-93 overexpression on TGF-β1-mediated EMT and fibrogenesis in HK2 cells. Taken together, Orai1 and miR-93 significantly impact on the progression of TGF-β1-mediated EMT and fibrogenesis in HK2 cells, and they may represent novel targets for the prevention strategies of fibrosis in the context of DN.

1. Introduction

One of the major microvascular complications of diabetes is diabetic nephropathy (DN), and it is a leading cause of death among diabetic patients.[1] It is characterized by excessive accumulation of ECM and renal interstitial fibrosis (RIF).[2] Recently, it has been recognized that fibroblasts deriving from renal tubular epithelium EMT lead to renal fibrosis.[3] EMT is characterized by the increased expressions of the mesenchymal markers, α-smooth muscle actin (α-SMA), vimentin, S100A4, and the decreased expression of the epithelial marker E-cadherin in tubular epithelia cells.[4] Previous studies have reported that one of the major and classic pathways leading to renal fibrosis is TGF-β1/Smad signaling pathway.[5] After activation, this signaling pathway can lead to the transcription of TGF-β1 target genes.[3,6] TGF-β1 can directly promote the production of ECM and the transdifferentiation of tubular epithelial cells to fibroblast.[6]

Increasing studies demonstrated that TGF-β1 mediates its effects by regulating the levels of some miRNAs, which have rapidly emerged as important regulatory molecules.[7,8] The role of many miRNAs such as the miR-29 family, the miR-200 family, miR-21 and miR-192 is well established in DN.[8] These miRNAs have been demonstrated to interact with and modulate various components of the TGF-β1-induced fibrotic programme. Moreover, in other cell types, the effect of TGF-β1 has also been confirmed.[9] Kato et al. report that TGF-β1 induces a decrease in miR-215 and an increase in miR-192 in mesangial cells, which is closely related to up-regulation of collagen I.[10] In the kidneys of diabetic mice, down-regulation of miR-192 dramatically increases ZEB1/2 and decreases expressions of fibronectin, collagen and TGF-β.[11] More recently, Wang et al. also reported TGF-β1 decreases expressions of the miR-29 family, leading to promoting expressions of ECM components.[12]

In this study, our findings showed that the miR-93 level is significantly reduced in renal tissues from patients with type 2 diabetes mellitus (T2DM) associated renal fibrosis compared with control subjects. In addition, miR-93 were confirmed to directly target Orai1 gene. The functional analysis demonstrated that knockdown of miR-93 enhanced expression of Orai1 and

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aggravated the profibrogenic effects of TGF-β1 on HK2 cells. Introduction of miR-93 decreased the Orai1 expression, and in turn suppressed TGF-β1-mediated EMT and renal fibrogenesis. Based on above results, miR-93 may represent a novel target for the prevention strategies of renal fibrosis in the context of DN.

2. Material and methods

2.1. Patients and tissue samples

The healthy control kidney tissues were obtained from patients who underwent nephrectomy as part of their tumor treatment (n = 10), and detected by renal needle biopsy. All healthy control patients have normal urine routine test, normal renal function, and normal blood pressure. The DN tissues were obtained from DN patients with T2DM who underwent diagnostic procedures (n = 10), and also detected by renal needle biopsy. All samples were collected from the Department of Nephrology, First Affiliated Hospital of Harbin Medical University. Written informed consent was obtained from all patients, and the studies were approved by the Ethics Committee of First Affiliated Hospital of Harbin Medical University.

2.2. Cell culture

Immortalized HK2 cells were cultured in DMEM/F12 containing 10% FBS (Gibco, USA), 1.20 g/L sodium bicarbonate, 1% penicillin-streptomycin at 37 °C in 5% CO_2.

2.3. Transient transfection

HK2 cells were transfected with miR-93 mimic or inhibitor (RiboBio, China) by using Lipofectamine 3000 (Thermo, USA) according to the manufacturer's protocols. After transfection, cells were treated with or without TGF-β1 for 24 h, and then harvested. The Orai1 expression was quantified by Western Blot and qRT-PCR, respectively. Total RNA prepared from HK2 cells was used to check the levels of miR-93, Fibronectin, Collagen IV, S100A4, vimentin, α-SMA, E-cadherin, Snail, Slug and Twist by qRT-PCR.

2.4. RNA extraction and qRT-PCR

Total RNA of tissues and HK2 cells was extracted for analyzing miRNA (Qiagen, USA) and mRNA (Axygen, USA) levels according to the manufacturer's protocols. For quantification of miR-93, miR-20a, miR-17, miR-106a and miR-519d, the TaqMan MicroRNA Reverse Transcription Kit and TaqMan miRNA assay (Qiagen, USA) were used to perform reverse transcription and PCR. U6 was used as the internal control. The gene expressions of Orai1, Fibronectin, Collagen IV, α-SMA, vimentin, S100A4, E-cadherin, Snail, Slug and Twist were determined by using the SYBR Green PCR kits (Takara, Japan). GAPDH was used as an internal reference. Each sample was assessed in triplicate. The used primers were listed in Table 1.

2.5. Western blot analysis

HK2 cells were lysed in RIPA buffer (Thermo, USA) with protease inhibitor cocktail (Millipore, USA). The protein concentration was quantified by BCA Kit (Thermo, USA), and proteins were separated by 10% SDS-PAGE (Bio-rad, USA), and then transferred onto the PVDF membrane (Millipore, USA). The membranes were blocked in 5% non-fat dry milk at room temperature (RT) for 2 h and probed overnight at 4 °C with primary antibodies including Orai1 antibody and phosph-Smad3 antibody (1:1000, Abcam, USA). Next, the membranes were wash three times and incubated with the second antibody (1:1000 dilution, CST, USA) for 2 h at RT. Incubation with monoclonal mouse α-tubulin antibody (1:1000 dilution; CST, USA) was performed as the loading control. The proteins were visualized using ECL western blotting detection reagents (Millipore, USA). The densitometry of the bands was quantified by using the Image J software (USA).

Table 1
Sequence of primers for RT-PCR.

Gene	Primer Sequence
Orai1	F: 5′-GACTGGATCGGCCAGAGTTAC-3′
	R: 5′-GTCCGGCTGGAGGCTTTAAG-3′
Fibronectin	F: 5′-CGGTGGCTGTCAGTCAAAG-3′
	R: 5′-AAACCTCGGCTTCCTCCATAA-3′
Collagen IV	F: 5′-GGACTACCTGGAACAAAAGGG-3′
	R: 5′-GCCAAGTATCTCACCTGGATCA-3′
α-SMA	F: 5′-AAAAGACAGCTACGTGGGTGA-3′
	R: 5′-GCCATGTTCTATCGGGTACTTC-3′
vimentin	F: 5'-GACGCCATCAACACCGAGTT-3'
	R: 5'-CTTTGTCGTTGGTTAGCTGGT-3'
S100A4	F: 5′-GATGAGCAACTTGGACAGCAA-3′
	R: 5′-CTGGGCTGCTTATCTGGGAAG-3′
E-cadherin	F: 5′-CGAGAGCTACACGTTCACGG-3′
	F: 5′-GGGTGTCGAGGGAAAAATAGG-3′
Snail	F: 5′-TCGGAAGCCTAACTACAGCGA-3′
	F: 5′-AGATGAGCATTGGCAGCGAG-3′
Slug	F: 5′-CGAACTGGACACACATACAGTG-3′
	F: 5′-CTGAGGATCTCTGGTTGTGGT-3′
Twist	F: 5′-GTCCGCAGTCTTACGAGGAG-3′
	F: 5′-GCTTGAGGGTCTGAATCTTGCT-3′
U6	F: 5′-CTCGCTTCGGCAGCACA-3′
	F: 5′-AACGCTTCACGAATTTGCGT-3′
GAPDH	F: 5'-CACCCACTCCTCCACCTTTG-3'
	R: 5'-CCACCACCCTGTTGCTGTAG-3'

2.6. Dual-luciferase reporter assay

The 3′-UTR was cloned into the luciferase reporter pGL3-control vector (Promega, Madison, WI) to construct pGL3-Orai1 vector. Overnight, HK2 cells were transfected with pGL3-Orai1-3′UTR wild-type or mutant reporter plasmid, miR-93 inhibitor, inhibitor-NC, miR-93 mimic, miR-NC or pRL-TK Renilla luciferase reporter (Promega) by using Lipofectamine 2000 (Invitrogen). Finally, luciferase activities were quantified by using the Dual-Luciferase reporter system (Promega) according to the manufacturer's protocols. Moreover, firefly luciferase activities were normalized to renilla luciferase activities.

2.7. Statistical analysis

Experiments were repeated at least three times. Values are expressed as mean ± SEM. Statistical analysis was determined by a paired two-tailed t test or one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison post hoc test. Data were evaluated for statistical significance by analysis using one-way. $P < 0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using GraphPad Prism 5.0 (USA).

3. Results

3.1. The level of miR-93 was decreased and the Orai1 expression was increased in human kidneys with fibrosis and TGF-β1-stimulated HK2 cells

There were 6 female and 4 male DN patients, and the mean age was 54 ± 10 years. The mean diastolic blood pressure was

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