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miR-182 enhances acute kidney injury by promoting apoptosis involving the targeting and regulation of TCF7L2/Wnt/β-catenins pathway

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ABSTRACT

Acute kidney injury (AKI) is a sudden decay in renal function leading to increasing morbidity and mortality. miR-182 has been reported to be actively involved in kidney diseases. However, the function and molecular mechanism of miR-182 in AKI still need to be elucidated. The levels of serum creatinine (SCr), blood urea nitrogen (BUN), and urine Kim-1 in I/R-induced rat AKI model were detected by a Beckman Autoanalyzer. miR-182 and transcription factor 7-like-2 (TCF7L2) mRNA expression were measured by qRT-PCR. Flow cytometry and caspase-3 colorimetry analysis were performed to determine NRK-52E cell apoptosis. Bioinformatics and dual-luciferase reporter were used to identify the interaction between miR-182 and TCF7L2. miR-182 expression was increased in both I/R-induced rat models and hypoxia-treated NRK-52E cells, and miR-182 overexpression stimulated the apoptosis of hypoxia-induced NRK-52E cells. Dual-luciferase analysis disclosed that TCF7L2 was a target of miR-182. TCF7L2 suppressed hypoxia-induced apoptosis in NRK-52E cells, and the inhibitory effect of TCF7L2 on cell apoptosis could be reversed with miR-182 restoration. Moreover, the activity of Wnt/β-catenin signaling pathway was promoted following overexpression of TCF7L2 in NRK-52E cells with hypoxia treatment, and this effect was greatly attenuated by the increased miR-182 expression. Finally, in vivo experiment also validated the alleviation of miR-182 inhibitor on I/R-induced kidney injury and apoptosis via regulating TCF7L2/ Wnt/β-catenin pathway. miR-182 exacerbated AKI involving the targeting and regulation of TCF7L2/ Wnt/β-catenin signaling, unveiling a novel regulatory pathway in ischemia-reperfusion injury and elucidating a potential biomarker for AKI treatment.

1. Introduction

Acute kidney injury (AKI), defined as a rapid decrease of kidney function within 48 h, has troubled more than 13.3 million individuals and led to about 1.7 million deaths per years (Mehta et al., 2015). Pathophysiological changes, such as tubular or epithelial cell injury, vascular dysfunction, and inflammatory response, have been elucidated to participate in the initiation and progression of AKI via the regulation of different transcriptional factors and signaling pathways (Manne et al., 2015; Ratliff et al., 2013). Ischemia-reperfusion injury (IRI) has been regarded as the main etiology for the occurrence of AKI, especially for the patients who are undergoing cardiac surgery, kidney transplantation, and renal vascular obstruction (Mehta et al., 2007). Despite tremendous development of novel therapeutic approaches for AKI in clinical, there was still a lack of effective treatment to cure this disease (Zuk and Bonventre, 2016). Thus, it is of great value to understand the possible molecular mechanism involved in AKI pathogenesis for improving kidney injury management, detection and therapy.

MicroRNAs (miRNAs), a class of endogenous conserved non-coding RNAs about 22- to 25-nucleotide (nt) in length, have been widely known to be implicated in biological progresses via negatively fine-tuning target mRNA expression at post-transcriptional level (Yu and Ho, 2015). To date, the attendance of miRNAs in various cells or tissues has been extensively reported, and many of them have the potential to be the clinical biomarkers for kidney injury (Kito et al., 2015). Several miRNAs have been clarified in diverse types of AKI (Fan et al., 2016; Liu et al., 2016). For instance, overexpression of microRNA-494 contributed to kidney injury in ischemia/reperfusion (I/R)-induced rat model through inducing renal apoptosis and increasing inflammatory mediators level by attenuating the expression of activating transcription factor 3 (ATF3) (Lan et al., 2012). Up-regulation of miR-423–5p enhanced endoplasmic reticulum (ER) and oxidative stress, suppressed proliferation, and exacerbated apoptosis in hypoxia/reoxygenation (H/R)-induced HK-2 cells (Yuan et al., 2017).

miR-182, located in the region of chromosome 7q32.2 of human genome, is profusely expression in the retina and human embryonic

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stem cells (Wei et al., 2015; Xu et al., 2007). Accumulated studies have verified that abnormal expression of miRNA-182 is actively involved in plenty of pathophysiological processes, such as cancer invasion (Spitschak et al., 2017), neuronal development and maturation (Wang et al., 2017), granulocytic differentiation (Wurm et al., 2017), as well as osteogenesis (Kim et al., 2012). Interestingly, miR-182 was identified as a molecular regulator of post-transplantation AKI, and miR-182 expression reached a peak at 24 h after IRI in renal mouse model, offering the possibility of miR-182 as novel biomarker and target candidate in addressing AKI (Cui et al., 2016; Wilflingseder et al., 2014). Moreover, inhibition of miR-182 was disclosed to ameliorate kidney function and morphology in an animal model of AKI (Wilflingseder et al., 2016). This study was designed to investigate the detailed mechanism of miR-182 in AKI.

2. Materials and methods

2.1. I/R-induced rat AKI model

Same batch of Sprague Dawley (SD) male rats (250 ± 20 g) were purchased from the Shanghai Laboratory Animal Center. Rats were anesthetized with 50 mg/kg pentobarbital through intraperitoneal injection, and suffered from bilateral renal artery occlusion for about 30 min. Following ischemia, the clamps were released for reperfusion. Rats in sham group received the same operation without artery clamping. To investigate the role of miR-182, intravenous injection with miR-182 inhibitor (anti-miR-182) at a dose of 40 mg/kg via tail was performed in rats in I/R and Sham group 24 h prior to ischemic surgery. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animal with approval of the Ethics Committee of Huaihe Hospital. About 24 h after I/R surgery, rats were killed for collecting the blood, urine, and kidney tissues.

2.2. Cell culture and transfection

Rat renal proximal tubular cell line (NRK-52E) and 293T cells were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and grown in DMEM medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) in a humidified atmosphere of 5% CO_2 at 37 °C.

miR-182 mimics (miR-182), scrambled control (miR-con), miR-182 inhibitor (anti-miR-182), and inhibitor control (anti-miR-con) were all synthesized by GenePharma Co. Ltd. (Shanghai, China). The full-length transcription factor 7-like 2 (TCF7L2) sequences were cloned into pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) to construct recombinant TCF7L2-overexpressing plasmid pcDNA-TCF7L2 (TCF7L2). NRK-52E cells were transfected with 20 nM miRNAs or 0.5 μg plasmids with Lipofectamine 2000 reagent (Invitrogen) for 24 h. For the monitoring of transfection efficiency, miR-182 expression was determined following the transfection of miR-182 or miR-182 inhibitor, and TCF7L2 expression was measured after transfecting with TCF7L2 in Hypoxia-induced NRK-52E cells.

2.3. Construction of hypoxia-induced NRK-52E cell model

Briefly, transfected NRK-52E cells were collected into the six-well plate with a density of 1×10^6/well and cultured in serum-free medium at 37 °C with 1% O_2/5% CO_2/94% N_2 for 4 h in Hypoxia Modular Incubator Chamber (Billups-Rothenberg Inc, San Diego, California, USA). Next, cells were switched to complete medium and incubated in the normoxic conditions for additional 24 h.

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

Total RNA in kidney tissues and NRK-52E cells were isolated by Trizol reagent (Invitrogen). RNA concentration and integrity were detected by spectrophotometer (UNICO, Dayton, Ohio, USA) and denaturing agarose gel electrophoresis (Bio-Rad, Hercules, CA, USA). After that, 10 ng of RNA were collected for cDNA synthesis using miRNA First-Stand cDNA Synthesis Kit (GeneCopoeia, Guangzhou, China) or High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). RT-PCR reactions were carried out by TaqMan™ Advanced microRNA Assay (Thermo Fisher Scientific) and Universal SYBR Green PCR Kit (Takara, Dalian, China) on an Applied Biosystems 7500 Real-time PCR Systems (Applied Biosystems, Foster City, CA, USA). The expression of miR-182 and TCF7L2 mRNA were respectively normalized to U6 and GAPDH. The reverse-transcription primer for miR-182: GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CAG TGTG. The specific primers for qRT-PCR were shown as below: miR-182, 5′- TTT GGC AAT GGT AGA ACT CAC ACT -3′ (forward) and 5′- GCG AGC ACA GAA TTA ATA CGA C -3′ (reverse); U6, 5′- CGC TTC GGC AGC ACA TAT ACT A -3′ (forward) and 5′- CGC TTC ACG AAT TTG CGT GTC A -3′ (reverse); TCF7L2, 5′- TGG AAA CTA AGG CGT GAG GGG -3′ (forward) and 5′- GGG CTG ATG GG TTG AAT GTG -3′ (reverse); GAPDH: 5′- TAT GAT GAT ATC AAG AGG GTA GT-3′ (forward) and 5′- TGT ATC CAA ACT CAT TGT CAT AC -3′ (reverse).

2.5. Cell apoptosis assays

NRK-52E cell apoptosis assay was determined using Annexin V-FITC/PI Apoptosis Detection Kit (BestBio, Shanghai, China) according to the manufacturer's instructions. Briefly, cells were digested with EDTA-free trypsin (Gibco), washed with PBS, and resuspended in 1 × binding buffer with a density of 1×10^6 cells/well, followed by addition of equal amount of Annexin V-FITC and propidium iodide (PI). Apoptotic cells (FITC-positive and PI-negative cells) were observed by Flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

2.6. Caspase-3 activity assay

Caspase-3 activity was evaluated by Caspase-3 Colorimetric Assay Kit (Biovision, Shanghai, China) according to manufacturer's instructions. The OD values of samples were measured at a wavelength of 405 nm using a microplate reader (Bio-Tek, Vermont, USA).

2.7. Western-blot assay

NRK-52E cells were subjected to RIPA lysis buffer for protein extraction (Solarbio, Beijing, China). The equal protein extracts were divided by SDS-PAGE, and then electrotransferred to nitrocellulose (NC) membranes (Millipore, Billerica, MA, USA). Then, the membranes were blocked with 5% skimmed milk powder solution for 2 h, followed by incubated with mouse specific antibodies against active caspase 3, total caspase 3, Bcl-2, Bax, β-catenin, c-myc, cyclinD1, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and TCF7L2 (synthesized by Genecreate, Wuhan, China) overnight at 4 °C. After washing the membranes with TBST buffer for three times, HRP-conjugated goat-anti-mouse secondary antibody (Santa Cruz Biotechnology) were added for another 1 h of incubation. All target proteins were quantified by comparing with standardized β-actin. The protein bands were visualized by chemiluminescence system (Pierce, Rockford, Illinois, USA).

2.8. Dual-luciferase reporter assay

TCF7L2 3′-untranslated region (UTR) sequences containing wild-type or mutated miR-200c binding sites were cloned into psiCHECK™-2 luciferase plasmid (Promega, Madison, WI, USA) to generate wild-type TCF7L2 (TCF7L2-WT) or mutated TCF7L2 (TCF7L2) reporter. 293T cells were transfected with reporter plasmids along with miR-182 or miR-con. Finally, the activity of luciferase reporters were measured