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Down-regulation of miR-34a alleviates mesangial proliferation *in vitro* and glomerular hypertrophy in early diabetic nephropathy mice by targeting GAS1

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Aims—Diabetic nephropathy (DN) is a major diabetic complication characterized by mesangial proliferation and glomerular hypertrophy. MicroRNAs might play an important role in these pathological processes. The aim of this study is to examine the possible association of miR-34a as one of the microRNAs with DN and underlying mechanisms *in vitro* and *in vivo*.

Methods—According to previous results of microarray which compared the different microRNAs between diabetic and normal control mice, miR-34a was chosen and its expression was detected by qRT-PCR. Cell viability was then assessed using Cell Counting Kit-8 (CCK8) and 5-ethynyl-20-deoxyuridine (EDU) incorporation. Antagomir was injected in db/db mice to down regulate miR-34a. Average diameter of glomeruli was analyzed by periodic acid-Schiff (PAS) stain of kidney. Luciferase gene report assay was then performed to identify the target gene of miR-34a. Additional immunoblotting and immunohistochemical analyses were implemented to verify the expression level of growth arrest-specific 1 (GAS1).

Results—MiR-34a expression level was increased under high glucose condition *in vitro* and *in vivo*. Down-regulation of miR-34a inhibits mice mesangial cells (MMCs) proliferation *in vitro* and alleviates glomerular hypertrophy *in vivo*. GAS1 was proved to be the target of miR-34a through

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luciferase report. Moreover, up-regulation of GAS1 expression was observed in the presence of miR-34a antagomir as compared withmiR-34a antagomir-NC in high-glucose-treated MMCs and db/db mice, respectively.

Conclusions—MiR-34a regulated mesangial proliferation and glomerular hypertrophy by directly inhibiting GAS1 in early DN.

Keywords

Diabetic nephropathy; Glomerular hypertrophy; miR-34a; GAS1

1. Introduction

Diabetic nephropathy (DN) is one of the severe complications of diabetes. It is characterized by mesangial cell proliferation, extracellular matrix (ECM) accumulation, glomerulosclerosis and always leads to end-stage kidney diseases (ESKDs) (Eid et al., 2013; Sun, Su, Li, & Wang, 2013). Evidences suggest that proliferation of mesangial cells is critical in the initiation and progression of DN (Liu et al., 2012; Wolf & Ziyadeh, 1999). In recent years, several studies have identified the regulatory roles of some factors in proliferation of mesangial cells (Onozaki et al., 2004; Zhang et al., 2012), but the detailed mechanisms involved in the progression of DN have not been fully understood.

MicroRNAs (miRNAs) are a group of small non-coding single-stranded RNAs which have about 17–24 nucleotides in length. It is estimated that more than two thirds of total human mRNAs are targeted by miRNAs (Fernandez-Hernando, Ramirez, Goedeke, & Suarez, 2013). Recently, it has been indicated that miRNAs are highly involved in pathology of diabetes and its relevant renal injuries (Conserva, Pontrelli, Accetturo, & Gesualdo, 2013; Zhang et al., 2009; Zhang et al., 2012). Previous microarray results suggested that miR-34a was up-regulated in DN (Chen et al., 2012). In some other studies, miR-34a is usually closely related with cell proliferation (Chen et al., 2011; Dang, Luo, Rong, & Chen, 2013). So, we hypothesize that miR-34a might affect mesangial cells proliferation in the pathological process of DN. Subsequently, we examine the mechanism of miR-34a regulation in DN in this study.

Growth arrest-specific 1 (GAS1) is identified as a putative tumor suppressor gene. GAS1 protein is a glycosyl-phosphatidyl-inositol (GPI)-anchored protein, which is overexpressed in growth-arrested embryonic mouse NIH/3 T3 fibroblasts and inhibits their proliferation by blocking the cell cycle G0/S transition (Evdokiou & Cowled, 1998; Stebel et al., 2000). GAS1 shows high homology to the glial cell=derived neurotrophic factor (GDNF) family receptor-α (Cabrera et al., 2006). GAS1 is also expressed in the kidney during development and under pathological conditions, but the function of GAS1 in the adult kidney is still not completely known.

In the present study, we aimed to investigate the critical roles of miR-34a in proliferation of mesangial cell under hyperglycemia and glomerular hypertrophy during early DN and to clarify the underlying mechanisms.

2. Materials and methods

2.1. Cell culture

Isolation of mice mesangial cells (MMCs) was performed according to the method described previously (Kim, Reddy, Lanting, Adler, & Natarajan, 2003). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum(FBS) and were kept in a humidified incubator that was maintained at 37 °C and supplied with 5% CO_2 and 95% air. Passages between 3 and 8 were used in all experiments.

2.2. Transient transfection

All of the transient transfections were performed with Lipofecta-mine 2000 Reagent (Invitrogen, USA) according to the manufacturer's recommendations. miRNA oligonucleotide transfections used in loss of-function experiment were performed according to an established protocol. Briefly, cells were seeded onto 6-well plates and were grown to 80% confluency. Next, a miRNA antagomir, or a matched miRNA negative control (Genepharma, Shanghai, China) was added to the culture media at a final concentration of 100 nM. After 6 h of transfection, the medium was replaced with RPMI-1640 containing 5.6 or 30 mM glucose, and cells were incubated for 48 h. Transfection efficiency (>90%) was measured by qRT-PCR.

2.3. In vitro cell proliferation assay

Cell viability was assayed by a colorimetric procedure, using the Cell Counting Kit-8 (Dojindo, Shanghai, China) according to the manufacturer's protocol. The absorbance at 450 nm was determined with a microplate reader. For each group, 5 duplicate wells were detected per experiment. To detect the exact proliferation rates of MMCs, an EDU (5-ethynyl-20-deoxyuridine) incorporation assay was executed with the Cell-Light™ EdU In Vitro Imaging Kit (Ribobio, Guangzhou, China) according to the producer's instructions. Briefly, cells treated as mentioned above were incubated with 10 μM EDU in RPMI-1640 medium for 2 h before fixation in 4% paraformaldehyde. After EDU staining, cell nuclei were stained with Hoechst 33342 and visualized under fluorescence microscopy (Leica, Germany). For each group, 3 random fields were photographed. The proliferation rate was calculated by the number of EDU-stained cells normalized by the number of Hoechst 33342-stained cells.

2.4. Glomerular morphological analysis

The sections of renal glomeruli from mice of each group were stained with Periodic acid-Schiff (PAS) and observed under microscope. The average diameter of glomeruli was measured with image analysis software (Image-pro plus, Version 5.1.0.20, Media Cybernetics, Inc). Glomerular tufts in a group were counted in nine different visual fields.

2.5. Luciferase reporter gene assay

Using target scan software, GAS1was predicted to be a target of miR-34a. The full length 3'-UTR of GAS1 was amplified by PCR from mice genomic DNA and cloned at the SacI and XhoI sites into luciferase reporter vector (Promega, Madison, WI, USA). The mutant or

WT construct of GAS1 3'UTR was generated by GuangZhou Ribobio Company in China. 293T cells were co-transfected with a reporter construct (pmiR-null Report plasmid, pmiR-GAS1 3'-UTR, pmiR-GAS1 3'-UTR-Mut) and miR-34amimic or miR-negative control. After 24 h of incubation, the luciferase activities were measured in a luminometer with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's recommendations.

2.6. Real-time quantitative PCR (qRT-PCR)

Total RNA, including microRNA, was extracted from cultured cells and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. miRNA qRT-PCR was performed using SYBR Premix ExTaq TM(Takara, Tokyo, Japan) according to the manufacturer's instructions and an Applied Biosystems 7500 Fast real-time PCR system, as reported previously (Bernardo et al., 2012). The PCR cycling conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 35 s. U6 snRNA (Applied Biosystems) was used as an endogenous normalization control. Individual samples were run in triplicate, and each experiment was repeated at least 3 times. Data analyses were performed using the comparative CT (ΔΔCT)method for calculating relative gene expression.

2.7. Western blot analysis

Western blotting was performed as described previously (van Roeyen et al., 2013). The following antibodies were used: anti-GAS1 antibody (1:100; Santa Cruz), anti-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) Antibody (1:3000; Sigma) was used as a loading control. The results were analyzed using Quantity One software (Bio-Rad, Richmond, Calif., USA).

2.8. Animals experiments

Male 4-week-old C57BL/KsJ type 2 diabetic db/db mice and heterozygote age-matched db/m mice were originally purchased from Model Animal Research Center of Nanjing University (Nanjing, China). Mice were kept in the Laboratory Animal Center of the Third Military Medical University under controlled temperature (20–22 °C), light (12-/12-h light/dark cycle) and humidity (50%–60%) and received food and water ad libitum. Db/db and db/m mice that were 8 weeks old were randomly subdivided into four experimental groups of 8 animals each: (1) control (untreated db/m mice) group, (2) untreated db/db group, (3) antagomir-34a-Negative Control (antagomir NC)-treated db/db group, and (4) antagomir-34a-treated db/db group. Db/db mice were injected with miR-34a antagomir or antagomir NC (80 mg/kg/day) (Genepharma, Shanghai, China) via the tail vein for three consecutive days. The mice injected with miR-34a antagomir or antagomir NC behave as the normal ones and did not exhibit any signs of discomfort. Four weeks later, the mice were sacrificed and the kidneys from the mice were collected as described previously (Park et al., 2007). One piece of kidney tissues was fixed by neutral formalin for immunohistochemical analysis, and the remaining tissues were stored at −70 °C for later use. All experiments were approved by the Animal Care and Use Committee of the Third Military Medical University and complied with the Declaration of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.