MiR-214 promotes renal fibrosis in diabetic nephropathy via targeting SOCS1

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Abstract

Purpose: To elucidate how miR-214 regulates the pathogenesis of diabetic nephropathy (DN).

Methods: The extent of fibrosis in DN mice kidneys was examined using Masson’s staining. Quantitative polymerase chain reaction (qPCR) was used to determine the levels of miR-214. Dual luciferase reporter assay was used to identify the target of miR-214. The expression of fibrosis marker proteins of high glucose-stimulated NRK-52E cells transfected with miR-214 was determined using western blotting.

Results: Fibrosis in renal tissue of DN mice was significantly increased and miR-214 was upregulated (p < 0.001). Suppressor of cytokine signaling 1 protein (SOCS1) was the target gene of miR-214, and overexpression of miR-214 promoted fibrosis (p < 0.05, p < 0.001). On the other hand, overexpression of SOCS1 inhibited this process, indicating that miR-214 promoted fibrosis via targeting SOCS1 (p < 0.001). Finally, inhibition of miR-214 ameliorated renal fibrosis in DN mice (p < 0.01, p < 0.001).

Conclusions: MiR-214 is upregulated in db/db DN mice kidney tissue; miR-214 regulates renal fibrosis in DN mice by targeting SOCS1.

Keywords: MiR-214, Renal fibrosis, Suppressor of cytokine signaling 1

INTRODUCTION

Diabetic nephropathy (DN), a common complication of diabetes, is considered to be a major cause of kidney failure [1,2]. With its increasing incidence, DN has become a global public health problem. In general, the unique pathological changes of DN, including mesangial cell (MC) proliferation, vascular mesangial expansion, and accumulation of abnormal extracellular matrix (ECM), leads to fibrosis of the glomerular sclerosis resistance index and ultimately to chronic renal failure [3]. These physiological changes dramatically decrease the quality of life, and even endanger the lives of patients [4].

MicroRNAs (miRNAs) are endogenously expressed and negatively regulated through targeting the 3'UTR regions of mRNAs [5]. miRNAs are essential for the pathogenesis of many human diseases, including cancer,
diabetes, diabetic complications such as DN, and other potential diseases [6-8]. Previous studies have reported that miR-214 is dramatically increased in the renal cortex of diabetic db/db mice, and suppression of miR-214 reduces the levels of collagen IV, SM22, and α-SMA, and partially recovers the levels of phosphatase and tensin homolog (PTEN) protein [9].

Cross-expression of PTEN and miR-214 protein under diabetic conditions suggests that miR-214 may be a new therapeutic target for DN [10]. Another study reported that miR-214 was increased in a kidney injury model, and genetic loss of miR-214 weakened interstitial fibrosis [9]. MiR-214 could facilitate fibrosis via the independent TGF-β signaling pathway in renal injury, but the molecular mechanism of this process is still unknown [11].

Suppressors of cytokine signaling (SOCS) proteins are important negative regulators that control the size and duration of the JAK/STAT signaling via inhibiting kinase activity, binding to STAT, and affecting the proteasome degradation pathway [12]. Members of the SOCS family include a C-terminal SOCS cassette, which is involved in the proteasome, a variable N-terminal domain, and a central SH2 domain [13].

This study showed that miR-214 was increased in kidney tissue of db/db DN mice and high glucose (HG)-stimulated NRK-52E cells, which targeted the SOCS1 gene. Overexpression of miR-214 promoted HG-stimulated NRK-52E cell fibrosis, but SOCS1 could inhibit this progress. Together, these results showed that miR-214 promoted HG-stimulated NRK-52E cell fibrosis through targeting SOCS1.

**EXPERIMENTAL**

**Db/db DN mouse model**

All animal experiments were conducted based on the “Guidelines for the Care and Use of Laboratory Animals” [14]. Six–8 weeks clean grade male db/db mice with background strain C57BL/KsJ were purchased from the Beijing Huakang Biotechnology Co. (Beijing, China). During the experiments, the mice were kept under constant temperature conditions, and were free to eat standard feed and drinking water, with the light/dark alternated every 12 h. The diabetic mice were then divided into two groups (n = 10/each group) involving the, (1) db/db DN group (db/db) and (2) the db/db DN group incubated with the miR-214 inhibitor (Invitrogen, Carlsbad, CA, USA). Age-matched db/m mice (db/m, n = 10) were used as controls. The kidney tissue was harvested and frozen at -80°C for subsequent western blotting and quantitative polymerase chain reaction (qPCR).

**Cell culture**

NRK-52E cells were cultured in collagen-coated, polystyrene T-25 culture flasks with DMEM medium (Gibco, Eggenstein, Germany). The cells were incubated in a constant temperature incubator at 37°C with 5% CO2. For the HG-stimulated NRK-52E cell, the cells were co-cultured with 30 mM D-glucose for 24 h until they reached approximately 90% confluence.

**Western blotting**

Isolated kidney tissue and NRK-52E cells were harvested and the protein was extracted. The total proteins were collected by centrifugation. Equal amounts of proteins were resolved by SDS-PAGE and immunoblotted with primary rabbit anti-SOCS1, anti-α-SMA, anti-TGF-β1 (1:500; Cell Signaling Technology, Boston, MA, USA), anti-Smad3, anti-nectin and anti-fibronectin, or anti-collagen I (1:200; Abcam, Cambridge, MA, USA). Goat anti-rabbit secondary antibody (1:3,000; BioRad, Hercules, CA, USA) labeled with horseradish peroxidase was then incubated with the blots. Protein binding was determined using a storage phosphor screen, and the ratio of the target protein to the β-actin bands of the internal reference protein was used to define the quantification of protein expression. All experiments were performed in triplicate.

**Luciferase activity assay**

The gene of SOCS1-3′-UTR, which binds to miR-214, was cloned into the psi-CHECK-2 Vector (Promega, Madison, WI, USA), and was designated as SOCS1 3′-UTR-WT. The SOCS1 3′-UTR-mutant (MUT) was obtained using a Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA, USA). For the luciferase assay, miR-214 mimics (Invitrogen) and normal control (NC) mimics were co-transfected with SOCS1 3′-UTR-WT or SOCS1 3′-UTR-MUT into NRK-52E cells. The luciferase activity was measured after 24 h of transfection.

**Masson’s stain**

db/db DN mouse kidney tissue was harvested and stained according to the following protocol. The section samples were hydrated in phosphate-buffered saline for 5 min at 25 °C and incubated in Bouin secondary fixative solution at 56 °C for 1 h. The slides were washed with
flowing H$_2$O for 1–2 min at room temperature to remove the yellow color, and the slides were cooled and briefly rinsed in deionized (DI) H$_2$O. Weigert's iron hematoxylin (hematoxylin A and hematoxylin B) was used for staining for 5–10 min at 25 °C. After washing with warm tap H$_2$O for 10 min and rinsing with DI H$_2$O, the cells were stained with Biebrich Scarlet-Acid Fuchsin for 5–15 min at 25 °C to stain the cytoplasm.

After washing three times with DI H$_2$O for 1 min each wash, the mixture was placed in phosphomolybdic acid-phosphoric acid for 10–15 min at room temperature to prepare the section for collagen staining. The slides were drained, and collagen was stained with Aniline Blue for 10 min and rinsed with DI H$_2$O, the cells were stained with Biebrich Scarlet-Acid Fuchsin for 5–15 min at 25 °C to stain the cytoplasm.

Expression of miR-214

Total RNA from kidney tissue and NRK-52E cells was isolated and extracted using TRIZol reagent according to the manufacturer’s instructions (Invitrogen) and quantitated using a NanoDrop2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). One μg of RNA was used in the reverse transcription reaction using a MicroRNA Reverse Transcription Kit (Takara, Shiga, Japan). qPCR was performed using SYBR Premix (BioRad) using specific primers to amplify miR-214, U6 was used as a reference gene. The miR-214 primer sequences were as follows: forward, 5'-CGGCGGACACGAGGACAGACA-3' and reverse, 5'-GGTGGCTCTTGAGGACAGGGTACCAGATATCCGACCAGCAACTCAGGC-3'; and the U6 primer sequences were as follows: forward, 5'-GTGCTCGGTTTCGGCAGACAGCATAATAC-3' and reverse, 5'-CCTTGCGCAGGGGCCATGCTAA-3'.

Statistical analysis

All the data are presented as mean ± SEM. All experiments were independently performed at least three times. Values of $p < 0.05$, $p < 0.01$, or $p < 0.001$ were regarded as statistically significant.

RESULTS

MiR-214 is upregulated in db/db DN mouse kidney tissue and HG-stimulated NRK-52E cells

To detect the relationship between expression levels of miR-214 with DN, we constructed DN mice. First, we measured the extent of kidney fibrosis in DN mice by Masson’s staining. The results showed that the degree of fibrosis of kidney tissue in DN mice was significantly higher than that of control mice (Figure 1 A). We then detected the expression of miR-214 in kidney tissue of db/db DN mice using qPCR. The results showed that the level of miR-214 was upregulated in DN mice compared to control mice (Figure 1 B), indicating that DN induced high expression of miR-214. To confirm this result, we constructed HG-stimulated NRK-52E cells and detected the expression level of miR-214 in these cells. The qPCR results indicated that miR-214 was also upregulated in HG-stimulated NRK-52E cells (Figure 1 C).

![Figure 1: MiR-214 is upregulated in kidney tissue of db/db diabetic nephropathy mice and high glucose-stimulated NRK-52E cells. (A) Masson’s staining was conducted to evaluate the level of renal fibrosis (left panel), and the extent of fibrosis. Student’s t-test: **$p < 0.001$** (right panel). (B and C) The reverse transcription-quantitative polymerase chain reaction detected the levels of miR-214 mRNA in db/db and db/m mice (B), or in high glucose-stimulated NRK-52E cells (C). Student’s t-test: "p < 0.001"

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<th>Gene</th>
<th>Forward (5’–3’)</th>
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<tr>
<td>miR-214</td>
<td>CGGGCGAACA</td>
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<td>GAGAGGGCAGAC</td>
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<td>U6</td>
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Target gene of miR-214 is SOCS1

According to nucleotide sequence alignment, we found that miR-214 paired with the 3'-UTR of the SOCS1 gene (Figure 2 A). To verify whether SOCS1 was the miR-214 target gene, we transfected NRK-52E cells with the normal control (NC)-mimic or miR-214-mimic, then co-transfected with SOCS1 wild-type (WT) or mutant (MUT) 3'-UTR, which were fused with a luciferase reporter. The results showed that compared with cells transfected with NC-mimic, transfection with the miR-214-mimic reduced the luciferase reporter gene expression with SOCS1 3'-UTR-WT, but not with SOCS1 3'-UTR-MUT (Figure 2 B). Together, these results showed that SOCS1 was the target gene of miR-214.

Figure 2: MiR-214 targets SOCS1 for downregulation. (A) Sequence alignment between miR-214 with the 3'-UTR of the SOCS1 gene. (B) The dual luciferase reporter assay determined the relative fluorescence activity in NRK-52E cells transfected with the normal control (NC)-mimic + SOCS1 3'-UTR-wild-type (WT), miR-214-mimic + SOCS1 3'-UTR-WT, NC-mimic + SOCS1 3'-UTR-mutant (MUT), and miR-214-mimic + SOCS1 3'-UTR-MUT. Student's t-test; *p < 0.001

MiR-214 promoted fibrosis of HG-stimulated NRK-52E cells

To determine the effect of miR-214 on NRK-52E cell fibrosis, we transfected the NC-mimic, miR-214-mimic, NC-inhibitor, or miR-214-inhibitor into NRK-52E cells cultured in HG, then detected the transfection efficiency by qPCR. The results showed that NRK-52E cells transfected with the miR-214-mimic had higher accumulation of miR-214, and cells transfected with miR-214-inhibitor had decreased miR-214 accumulation (Figure 3 A).

To further evaluate the effect of miR-214 on cell fibrosis, we determined the expression levels of the fibrosis marker genes, including SOCS1, α-SMA, TGF-β1, t-Smad3, CTGF, fibronectin, and collagen I by western blotting. The results showed that in cells transfected with the miR-214-mimic, the expression of the SOCS1 gene was decreased, but the expressions of fibrosis marker genes were significantly increased (Fig. 3B), indicating that the fibrosis of NRK-52E cells was induced. In contrast, the expression of the SOCS1 gene was upregulated in cells transfected with the miR-214-inhibitor, and the expression levels of fibrosis markers were downregulated (Figure 3 B). Together, these results indicated that miR-214 promoted HG-stimulated NRK-52E cell fibrosis.

Figure 3: miR-214 promoted fibrosis of high glucose-stimulated NRK-52E cells. (A) Reverse transcription-quantitative polymerase chain reaction was used to detect the level of miR-214 mRNA in NRK-52E cells transfected with the normal control (NC)-mimic or miR-214-mimic (left panel), or NC-inhibitor or miR-214-inhibitor (right panel). (B) Western blotting evaluated the expression levels of SOCS1 and fibrosis marker proteins, including α-SMA, TGF-β1, t-Smad3, CTGF, fibronectin, and collagen I in NRK-52E cells transfected with the NC-mimic or miR-214-mimic (upper panel), and NC-inhibitor or miR-214-inhibitor (bottom panel). β-actin accumulation was used as an internal reference control. Relative protein expression was quantified by Image_J software (right panel). Student’s t-test; **p < 0.05, ***p < 0.001

MiR-214 promoted HG-stimulated NRK-52E cell fibrosis by inhibiting SOCS1

SOCS1 is a target gene of miR-214 (Figure 2); therefore, to verify whether miR-214 promoted fibrosis of HG-stimulated NRK-52E cells by...
inhibiting the SOCS1 gene, we transfected the NC-mimic, miR-214-mimic, miR-214-mimic, or SOCS1 into HG-stimulated NRK-52E cells. We then detected the expression levels of fibrosis marker proteins by western blotting. The results showed that the level of SOCS1 was downregulated in the miR-214-mimic, but was returned to normal levels in the miR-214-mimic and SOCS1 co-transfected cells. At the same time, the reduced expression levels of fibrosis markers in miR-214-mimic cells were partially restored to normal levels in the miR-214-mimic and SOCS1 co-transfected cells (Figure 4). Together, these results indicated that the fibrosis of NRK-52E cells, which was promoted by miR-214, was inhibited by SOCS1.

**Figure 4: MiR-214 promoted high-glucose cultured NRK-52E cell fibrosis by inhibiting SOCS1.** Western blotting detected the expression levels of SOCS1 and fibrosis marker proteins, including α-SMA, TGF-β1, t-Smad3, CTGF, fibronectin, and collagen I in NRK-52E cells transfected with the normal control-mimic, miR-214-mimic, or miR-214-mimic + SOCS1 (left panel). β-actin was used as an internal reference control. The relative protein expression was quantified by Image J software (right panel). # or ## represent Student’s t-test; p < 0.01

**MiR-214 inhibitor improved renal fibrosis in DN mice**

To verify whether inhibition of miR-214 expression improved renal fibrosis in DN mice, we transfected NC-antagomir or miR-214-antagomir into NRK-52E cells, then detected the expression of miR-214 by qPCR to evaluate the transfection efficiency. The results showed that the expression of miR-214 was downregulated in cells transfected with the miR-214-antagomir (Fig. 5A).

To further detect the degree of fibrosis of transfected cells, we determined the expression levels of fibrosis markers by western blotting. The results showed that the levels of SOCS1 were significantly increased in miR-214-antagomir transfected cells, while the expressions of fibrosis markers were correspondingly decreased (Fig. 5B). Furthermore, we detected the degree of renal fibrosis in DN mice by Masson’s staining and by the renal interstitial injury score. The results showed that compared with the control mice, the degree of fibrosis of kidney tissues transfected with the miR-214-antagomir was significantly decreased, and the degree of renal interstitial injury was also reduced (Fig. 5C). Together, these results indicated that the miR-214 inhibitor improved renal fibrosis in DN mice.

**Figure 5: MiR-214 inhibitor improved renal fibrosis in diabetic nephropathy mice.** (A) Reverse transcription-quantitative polymerase chain reaction detected the levels of miR-214 mRNA in NRK-52E cells transfected with the NC-antagomir and miR-214-antagomir. (B) Western blotting detected the expression levels of SOCS1 and fibrosis marker proteins in NRK-52E cells transfected with the NC-antagomir and miR-214-antagomir. (C) Masson’s staining was conducted to detect the extent of renal fibrosis in NRK-52E cells transfected with the NC-antagomir and miR-214-antagomir (left panel), and the renal interstitial injury score was used to evaluate the extent of kidney fibrosis (right panel).

**DISCUSSION**

This study showed that expression of miR-214 was significantly upregulated in DN mice and HG-stimulated NRK-52E cells after targeting the SCOS1 gene. Our results indicated that while the expression of miR-214 was induced, the SOCS1 target gene was downregulated, ultimately leading to the upregulations of various fibrosis marker proteins and increasing renal fibrosis. This study showed that miR-214 was significantly upregulated in DN mice, and downregulated by the SOCS1 target gene. Many studies have reported that proteins of the SOCS family negatively regulate a variety of cytokine-mediated signaling pathways, which affect basic biological behaviors, including cell growth,
pulmonary, and apoptosis[15]. Recent studies have also reported that the mRNA and protein levels of the SOCS1/3 are significantly upregulated in the glomeruli during the early stages of diabetes, accompanied by enhanced phosphorylation of JAK2 and STAT1, indicating that SOCS1/3 may negatively regulate the JAK/STAT signaling pathway in DN[16]. In the present study, miR-214 downregulated the expression of SOCS1, which indicated that miR-214 may attenuate the negative regulation of SOCS1 protein on the JAK/STAT signaling pathway during DN.

DN is a major diabetic microvascular complication that leads to renal failure. The main characteristic pathological changes of DN are abnormal proliferation of MCs, excessive accumulation of the ECM, and significant thickening of the basement membrane [17,18]. Previous studies have reported that during diabetes, miRNA plays a crucial role in regulating extracellular matrix ECM synthesis and gene expression during MC hypertrophy [10]. It has been reported that miR-214 promotes the growth of diabetic MCs in vitro by targeting PTEN [11]. The results of the present study showed that inhibition of miR-214 reduced the expression levels of SM22, α-SMA, and type IV collagen, but restored the expression of PTEN, and decreased albuminuria and mesangial expansion [19]. PTEN overexpression improved miR-214-mediated MC hypertrophy, while the knockdown of PTEN mimicked MC hypertrophy. These results suggested that targeting miR-214 may be an effective strategy to alleviate diabetic kidney damage [20].

Glomeruli secrete diverse cytokines in a HG-stimulated environment, including in the presence of TGF-β1, causing increased accumulation of collagen, fibronectin, and laminin [21]. The extent of TIF is therefore reflected in the expression of collagens[22]. In the present study, the expression of collagen I was upregulated in cells transfected with the miR-214-mimic, indicating an increase in the degree of fibrosis in renal tissue. Previous studies reported that TGF-β1 is a determining factor in the pathogenesis of renal fibrosis, because it is significantly upregulated in many diseased kidney tissues with severe fibrosis, and activates the downstream Smad signaling pathway[23]. In the present study, under HG stimulation, the expression of miR-214 was increased, accompanied by the upregulation of fibrosis marker proteins including TGF-β1, p-Smad3, p-Smad3, and CTGF, indicating that miR-214 functions through the TGF-β1/Smad3 pathway to regulate renal fibrosis.

In addition, abnormal expression of miR-214 has been found in many human tumors, including colorectal cancer, nasopharyngeal carcinoma, gastric cancer, ovarian cancer, and breast cancer, which contributes insight into the pathogenesis and metastasis of these tumors [20]. Previous studies have reported that miR-214 induces cell survival and platinum compound resistance by targeting PTEN and activation of the Akt pathway [24]. MiR-214 regulates the proliferation, migration, and invasion of gastric cancer cells by targeting the PTEN gene [25]. In the present study, miR-214 was upregulated in DN mice; however, whether miR-214 has other target genes besides SOCS1 in the regulation of renal fibrosis in mice will require further study.

CONCLUSION

MiR-214 targeted the SOCS1 gene, which inhibited the cytokine signaling pathways. The SOCS protein is a key physiological regulator of innate and adaptive immunity. MiR-214 RNA targets the SOCS1 gene, to improve the degree of renal tissue fibrosis caused by DN. Overall, the results of this study provide a possible strategy for the treatment of DN.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the researchers listed in this article. All liabilities related with the content of this article will be borne by the authors. Weiwei Xi and Hua Li designed all the experiments and revised the paper. Xuming Zhao, Wumei Jun and Xueqin Fu formed the experiments, Wenjuan Jia and Minxi Lu wrote the paper.

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