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Review Article

miR-30c inhibits renal fibrosis in diabetic nephropathy by down-regulating ROCK2

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Abstract: miR-30c inhibits renal fibrosis in diabetic nephropathy (DN) by down-regulating ROCK2. Forty SPF SD male rats were selected and divided into groups A and B, with 20 rats in each group. Group A was the normal control group (CG), and group B (DN group) was the research group (RG) for modeling. The miR-30c and ROCK2 expression levels, the UAER expression and 24 h urine protein, and the changes of Cys-C, TGF-β1 and type IV collagen levels in the rats were observed. The miR-30c and ROCK2 expression levels in cells were detected. The effect of miR-30c and ROCK2 on the changes of UAER and 24 h urine protein, Cys-C, TGF-β1 and type IV collagen levels was observed. The miR-30c expression in the RG was dramatically lower than that in the CG ($P<0.05$), while the ROCK2 expression in the RG was dramatically higher than that in the CG ($P<0.05$). The expression levels of UAER, 24 h urine protein, Cys-C, TGF-β1 and collagen IV in the rats in the RG were dramatically higher than those in the CG ($P<0.05$). After transfection, when miR-30c increased, UAER, 24 h proteinuria and expression levels of Cys-C, TGF-β1, type IV collagen clearly decreased ($P<0.05$); when miR-30c decreased, UAER, 24 h proteinuria and expression levels of Cys-C, TGF-β1, type IV collagen clearly increased ($P<0.05$). After transfection, when ROCK2 increased, UAER, 24 h urine protein and expression levels of Cys-C, TGF-β1, type IV collagen clearly increased ($p<0.05$); when ROCK2 decreased, UAER, 24 h urine protein and expression levels of Cys-C, TGF-β1, type IV collagen clearly decreased ($P<0.05$). miR-30c with low expression in diabetic nephropathy can promote the occurrence of renal injury and accelerate the process of renal fibrosis by increasing ROCK2 protein.

Keywords: miR-30c, ROCK2, renal fibrosis in diabetic nephropathy

Introduction

Diabetes is a common disease in endocrinology and the most frequent chronic disease all over the world [1]. With the improvement of people's living standards and the continuous change of life style, the incidence of diabetes among the population is continuously increasing, and the diabetic patients are constantly showing a younger trend [2]. The median age of the diabetic group decreases year by year [3]. At present, diabetes has not yet been completely cured, and blood glucose reduction maintenance treatment can only be carried out through a long medication cycle [4]. Diabetes is not harmful, but easily causes a series of complications [5]. For example, diabetic nephropathy (DN) is a common chronic diabetic microvascular complication [6]. DN is renal injury caused by diabetes, and its main clinical manifestations are proteinuria, decrease of glomerular filtration rate, increase of blood pressure and renal failure [7]. However, renal fibrosis and other morphological changes can occur in advanced DN, which is the main cause of end-stage renal disease [8]. At the moment, clinical treatments for DN include controlling blood pressure and blood glucose, reducing blood lipids and a low protein diet, etc. Although some achievements have been made, its occurrence and development has not been completely blocked [9]. Therefore, exploring the pathogenesis and developmental mechanism of DN is still a hot topic in clinical research.

With intense investigation, more and more research points to miRNA as a breakthrough point for DN therapy. miRNA is a non-coding

Table 1. Primer sequence

	Upstream forward primer	Downstream reverse primer
miR-30c	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC-3'	5'-GCAACCCGTAGATCCGAACTT-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'

microRNA with a length of about 18-25 nucleotides, it plays an important regulatory role in cell growth, differentiation, proliferation and apoptosis [10]. In the past, mRNA treatment has been found to be effective in a variety of diseases [11]. Recent studies have shown that the miR-30 family plays a vital role in fibrosis process [12]. As a member of the miR-30 family, miR-30c is involved in regulating various target genes and signal pathways [13]. According to research, it can target and regulate the expression of transforming growth factor-β2 (TGF-β2) and SNAIL1 genes and participate in the occurrence and development of liver fibrosis [14]. Therefore, we suspect that miR-30c may also be involved in the process of renal fibrosis and mayb have a certain influence on DN. In order to verify our conjecture, these experiments will provide new ideas and directions for future clinical diagnosis and treatment by exploring the influencing mechanism of miR-30c on renal fibrosis in DN.

Materials and methods

Data of rats

Forty SPF SD male rats were bought from Liaoning Changsheng Biotechnology Co., Ltd. Certificate No. SCXK (Liao) 2015-0001. At 12 weeks of age, the body weight was 180±20 g, and they were divided into groups A and B, with 20 rats in each group. Group A was the normal control group (CG) and group B was the research group (RG) with DN modeling.

Cell data

Rat proximal tubular epithelial cell strain (NRK-52E cell) was bought from American Type Culture Collection (ATCC).

Disease modeling

Twelve healthy male SD rats were adaptively fed at a room temperature of 20-25°C and humidity of 50-60% for 1 week, and then we began to prepare the DN rat model. They were fasted 6-8 h before modeling, and received tail injection of STZmh/kg sterile citric acid-sodium citrate buffer. Seventy-two hours later, blood was collected from the tail vein to measure blood glucose of the rats, and 16.7 mmol/L or more was regarded as successful model building of diabetic rats. Qualitative detection of urinary protein was performed one week later. Positive urine protein is regarded as renal damage, i.e. DN rats have been successfully modeled.

This study has been approved by the animal experimental ethics committee of The First Affiliated Hospital of Xian Jiao Tong University.

Cell culture and transfection

The culturing with medium containing 10% fetal bovine serum was conducted in an incubator at 37°C, 5% CO_2. When the cell growth reached 80% confluence, cells were subcultured. Those in the miR-30c mimetic (miR-30c-mimics), miR-30c inhibitor (miR-30c-Inhibitor), miRNA mimetic negative control (miR-NC), ROCK2 interfering RNA (siRNA) (si-ROCK2), mimetic RNA (sh-ROCK2) and negative control (si-NC) were transfected with Lipofectamine™ 2000 kit (Invitrogen, Carlsbad, USA), and the operation steps were strictly in line with the kit instructions.

PCR

The total RNA was extracted from cells using TRIzol (RNA in blood was extracted according to the instructions of the blood total RNA rapid extraction kit). RNA concentration was determined by ultraviolet spectrophotometer, and then it was reversely transcribed to cDNA. Amplification was performed with RT-qPCR. Amplification conditions were as below: pre-denaturation at 95°C for 1 min, 95°C for 15 s, 60°C for 1 min, 40 cycles, 95°C for 15 s, 60°C for 1 min. Melting curve analysis was conducted at 95°C for 15 s. miR-30c employed U6 as internal reference, and relative expression was calculated by $2^{-\Delta\Delta qct}$ method. See in **Table 1**.

Western blot test

Protein was extracted from cultured cells in each group with RIPA buffer according to

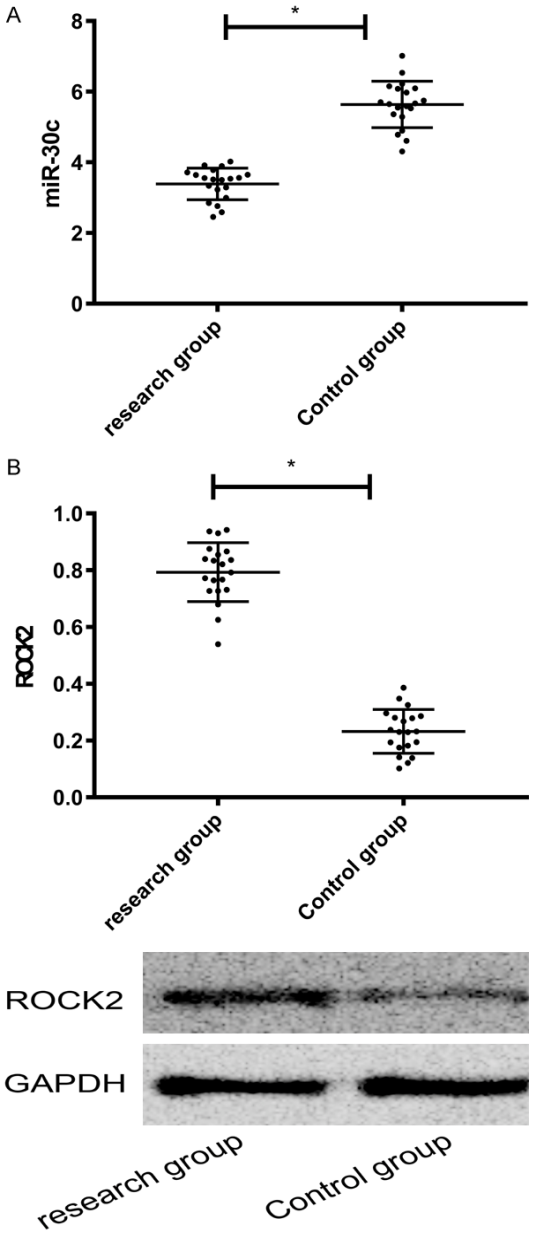

Figure 1. Expression of miR-30c and ROCK2 in rats was detected. A. miR-30c expression of rats in the two groups. B. ROCK2 expression of rats in the two groups.

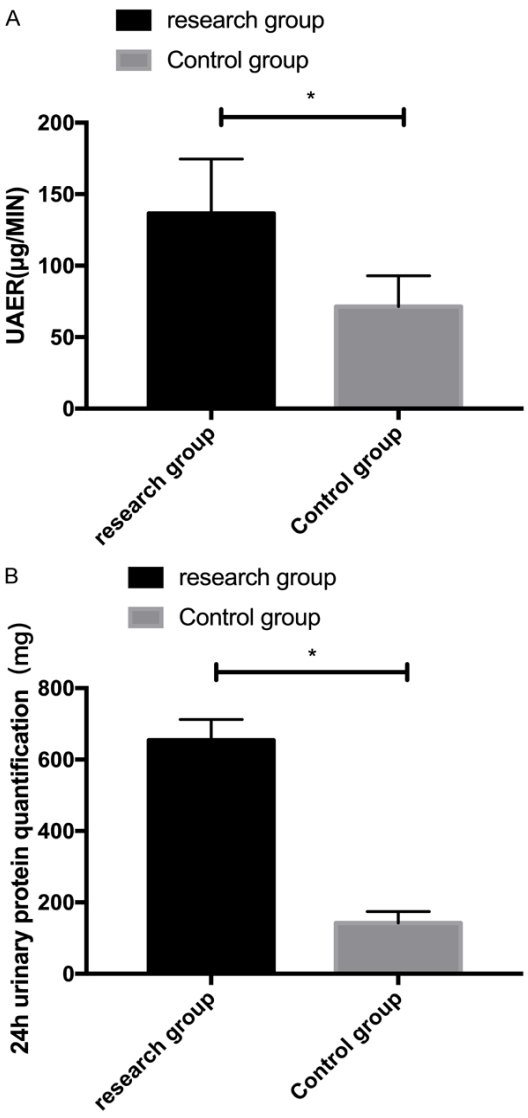

Figure 2. Expression of UAER and 24 h urine protein in rats. A. UAER expression of rats in the two groups. B. 24 h urinary protein expression of rats in the two groups.

instructions. The lysate was centrifuged for 20 min at 10,000× g, and then the supernatant was collected. The protein concentration was determined by BCA method (using bovine serum albumin as the standard). The same amount of protein was separated by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. The membrane was blocked for 1 h with 5% skim milk powder at room temperature, supplemented with Bax (1:1,000), Bcl-2 (1:2,000) and GAPDH (1:5,000) primary antibodies and incubated at 4 over night. Then, HRP labeled goat anti-mouse IgG secondary antibody (1:10000) was supplemented, and it was incubated it for 2 h. The protein bands on the membrane were developed in a dark room using the enhanced chemiluminescence reagent.

Detection methods

Urine albumin excretion rate (UAER) and 24 h urine protein were measured by a full-automatic biochemical analyzer (7600 type, Hitachi, Japan). Cys-C, TGF-β1 and IV collagen levels were detected by immunoturbidimetry. The kits

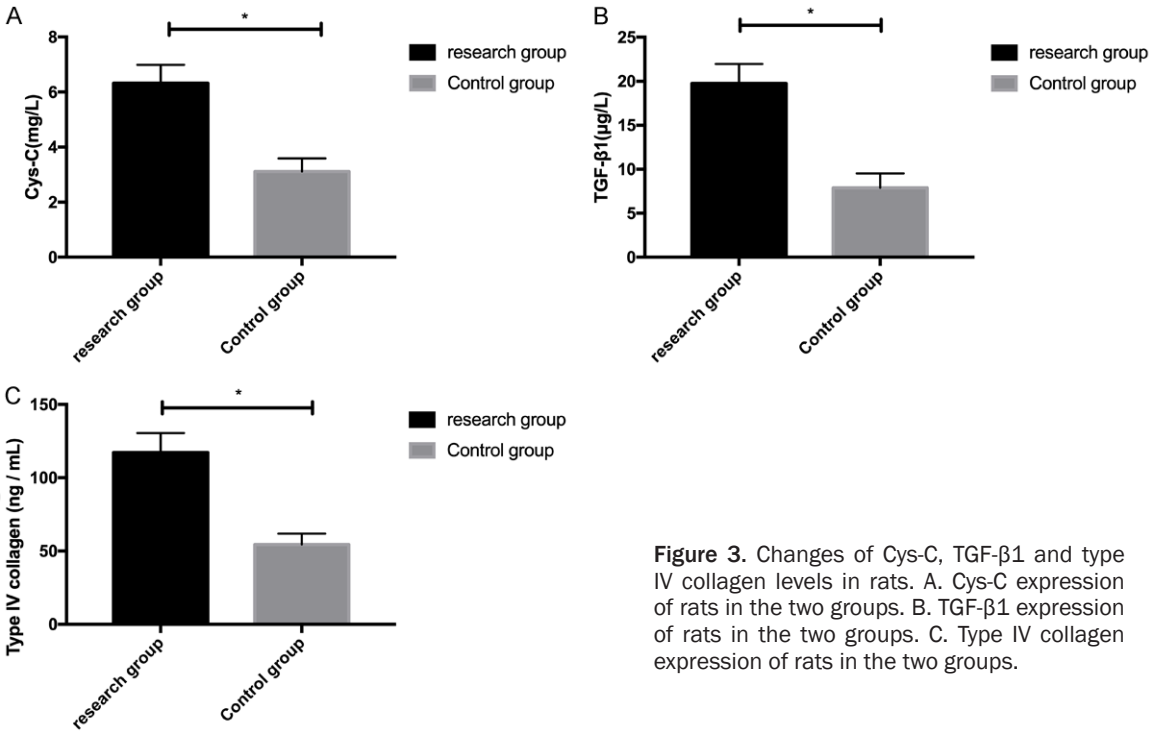

Figure 3. Changes of Cys-C, TGF-β1 and type IV collagen levels in rats. A. Cys-C expression of rats in the two groups. B. TGF-β1 expression of rats in the two groups. C. Type IV collagen expression of rats in the two groups.

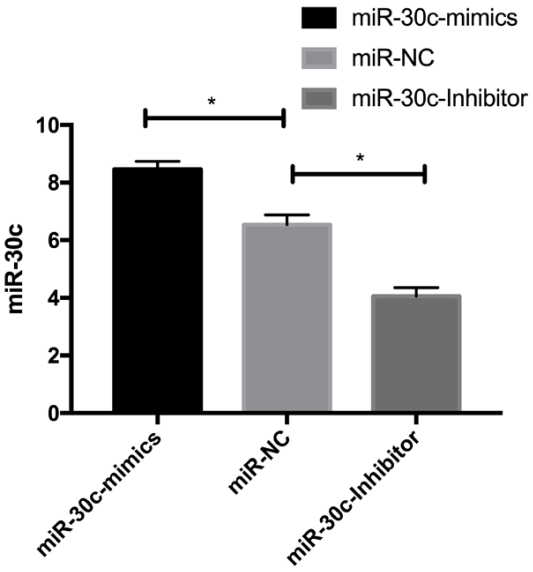

Figure 4. miR-30c expression in cells. *means P< 0.05.

were all provided by Beijing Century Ward Biotechnology Co., Ltd.

Targeted relationship verification

First, the target gene of miR-30c was predicted by TargetScanHuman72 online website (http://www.targetscan.org/vert_72/). The wild type ROCK2 (WT) fragment containing miR-30c binding site and its mutant seed sequence (MUT) (purchased from Shanghai Bioengineering Co., Ltd.) were inserted into the downstream of luciferase reporter gene of pmirGLO dual-luciferase miRNA target expression vector (Promega, Madison, WI, USA), respectively named ROCK2-WT and ROCK2-MUT. ROCK2-WT or ROCK2-MUT, miR-30c mimics and inhibitor were co-transfected with cells via Lipofectamine 2000. Forty-eight hours after transfection, the activity of luciferase was detected by dual-luciferase reporter analysis system (PROMEGA).

Statistical methods

The data results were analyzed and processed via SPSS 22.0 statistical software. All the results of this experiment were expressed in the form of (mean ± standard deviation). One-way analysis of variance (ANOVA) and LSD back testing were used for comparison among groups, and the difference was statistically significant at $P<0.05$.

Results

Expression of miR-30c and ROCK2 in rats was detected

The miR-30c and ROCK2 expression levels in the serum of rats in the two groups were detect-

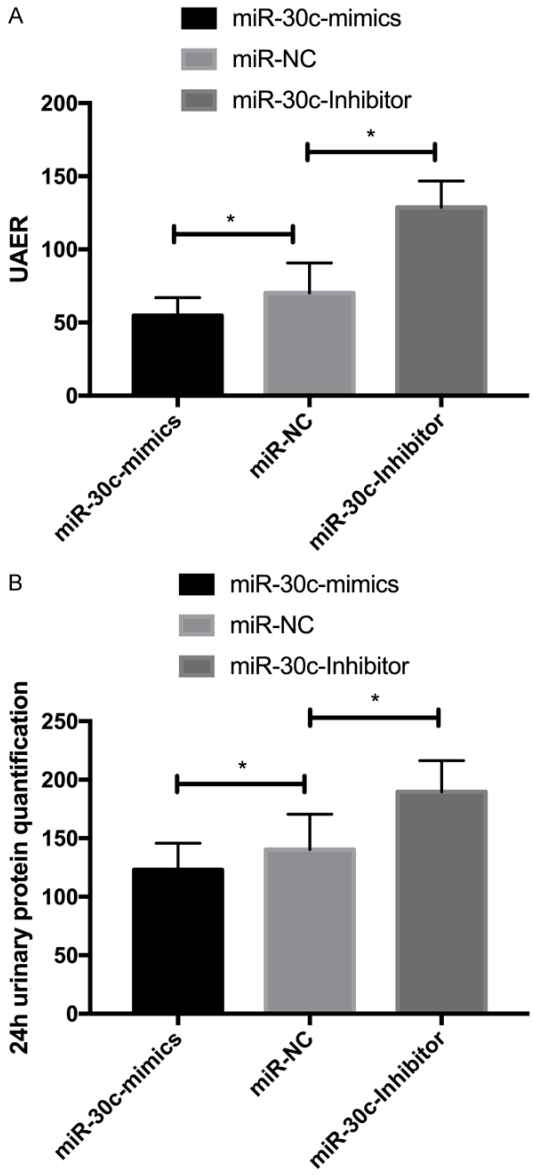

Figure 5. Effect of miR-30c on UAER and 24 h urine protein. A. Influence of miR-30c on UAER. B. Effect of miR-30c on 24 h urine protein.

ed. The results revealed that the miR-30c expression in the RG was dramatically lower than that in the CG, and the difference was statistically significant (P<0.05). The ROCK2 expression in the RG was dramatically higher than that in the CG, and the difference was statistically significant (P<0.05) (**Figure 1**).

Expression of UAER and 24 h urine protein in rats was detected

The UAER and 24 h urine protein expression levels in rats were detected. The results signified that the expression of both in rats of the RG was dramatically higher than that of the CG, and the difference was statistically significant (P<0.05) (**Figure 2**).

Changes of Cys-C, TGF-β1 and type IV collagen levels in rats

The changes of Cys-C, TGF-β1 and type IV collagen levels in rats were detected. The results indicated that their levels in rats in the RG were dramatically higher than those in the CG, and the difference was statistically significant (P< 0.05) (**Figure 3**).

miR-30c expression in cells

After cell transfection, miR-30c over-expression increased markedly (P<0.05), and inhibition of miR-30c decreased markedly (P<0.05) (**Figure 4**).

Effect of miR-30c on UAER and 24 h urine protein

After transfection, when miR-30c over-expression increased, UAER and 24 h urinary protein expression decreased dramatically (P<0.05). When miR-30c inhibition decreased, UAER and 24 h urinary protein expression increased dramatically (P<0.05) (**Figure 5**).

Effect of miR-30c on changes of Cys-C, TGF-β1 and type IV collagen levels

After transfection, miR-30c over-expression increased, and the levels of Cys-C, TGF-β1 and type IV collagen decreased dramatically (P< 0.05). Inhibition of miR-30c decreased, and the levels of Cys-C, TGF-β1 and type IV collagen increased dramatically (P<0.05) (**Figure 6**).

ROCK2 expression in cells

After cell transfection, the ROCK2 over-expression clearly increased (P<0.05), and ROCK2 inhibition clearly decreased (P<0.05) (**Figure 7**).

Effect of ROCK2 on UAER and 24 h urinary protein

After transfection, when ROCK2 over-expression increased, UAER and 24 h proteinuria expression increased significantly (P<0.05). When ROCK2 inhibition decreased, UAER and

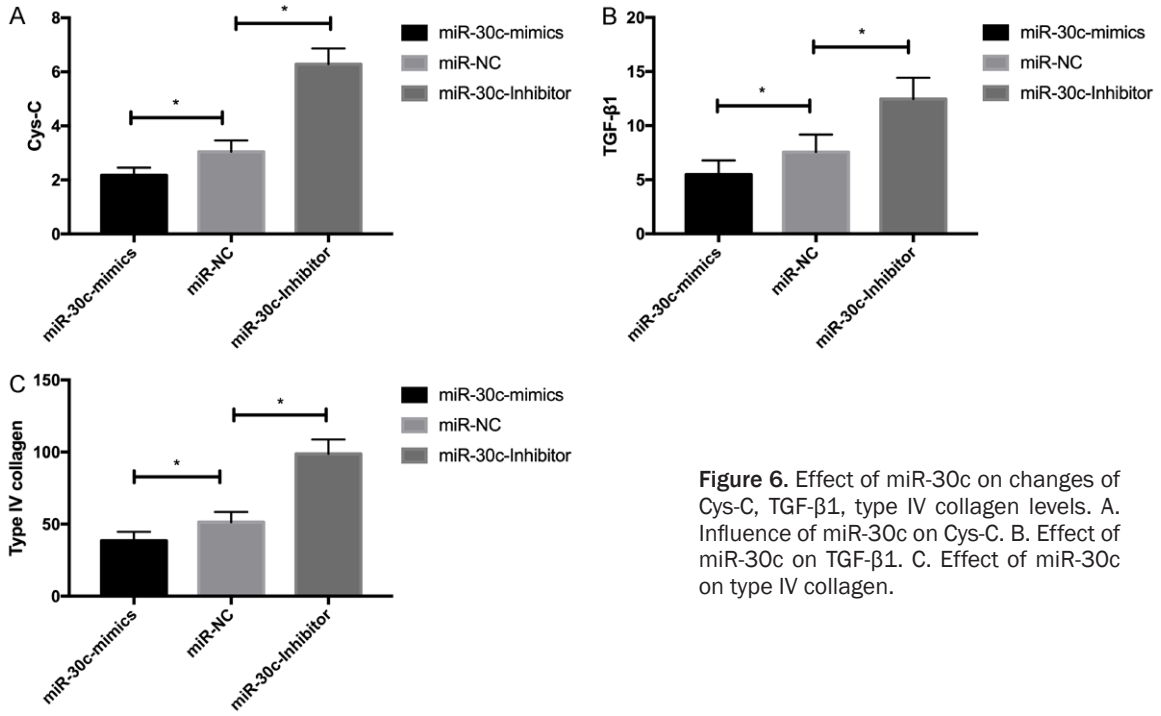

Figure 6. Effect of miR-30c on changes of Cys-C, TGF-β1, type IV collagen levels. A. Influence of miR-30c on Cys-C. B. Effect of miR-30c on TGF-β1. C. Effect of miR-30c on type IV collagen.

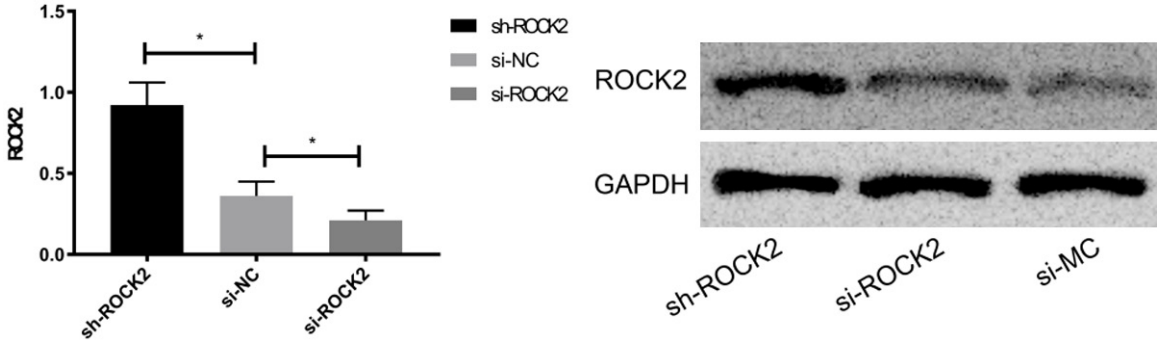

Figure 7. ROCK2 expression in cells.

24 h proteinuria expression decreased significantly (P<0.05) (**Figure 8**).

Effect of ROCK2 on changes of Cys-C, TGF-β1 and type IV collagen levels

After transfection, when ROCK2 over-expression increased, the levels of Cys-C, TGF-β1 and type IV collagen increased significantly (P< 0.05). When ROCK2 inhibition decreased, the levels of Cys-C, TGF-β1 and type IV collagen decreased significantly (P<0.05) (**Figure 9**).

Relationship between miR-30c and ROCK2

It was found that there was a binding site between miR-30c and ROCK2 through TargetScanHuman72 online website. The experimental results of dual-luciferase reporter enzyme assay showed that after transfection of miR-30c-mimics, the fluorescence activity of ROCK2-WT was inhibited (P<0.050). See **Figure 10**.

Discussion

DN is a complication caused by diabetic microangiopathy. As the number of diabetic patients increases year by year, the prevalence rate of DN also shows an increasing trend [15]. With the development of the disease, renal fibrosis poses a serious threat to the life and health of patients [16]. At present, the clinical treatment

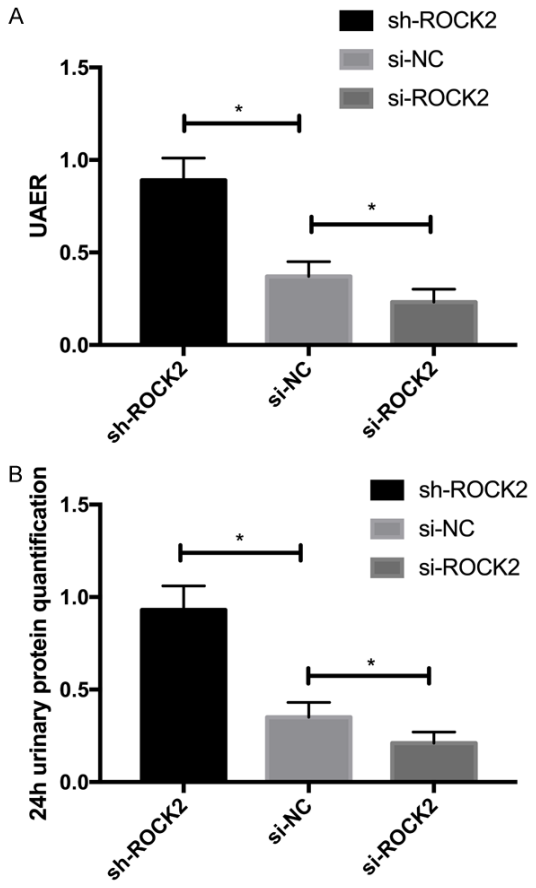

Figure 8. Effect of ROCK2 on UAER and 24 h urine protein. A. Effect of ROCK2 on UAER. B. Effect of ROCK2 on 24 h urine protein.

of DN is mainly through symptomatic treatment such as blood glucose control, blood pressure, blood lipids and antiplatelet aggregation, so as to reduce its progression [17]. There are still limitations in relieving clinical symptoms of patients and reducing urinary protein. Therefore, understanding the pathogenesis of DN is quite important for clinical diagnosis and treatment. In this research, miR-30c inhibited renal fibrosis in diabetic nephropathy by down-regulating ROCK2. The results were as follows.

The miR-30c expression in the RG was remarkably lower than that of normal mice (control group), while the ROCK2 expression was remarkably higher than that of normal mice. It suggested that miR-30c and ROCK2 might be involved in the occurrence and development of DN. However, the UAER, 24 h urine protein, Cys-C, TGF-β1 and collagen IV levels in the rats were detected, and the results signified that they were dramatically up-regulated in the rats in the RG. This result indicated that our modeling was successful, and that UAER, 24 h urine protein and Cys-C, TGF-β1, and type IV collagen might affect the formation of renal injury and fibrosis. Moreover, due to the abnormal expression of miR-30c and ROCK2 in DN, we speculated that the two affected the changes of UAER, 24 h urine protein and Cys-C, TGF-β1, and type IV collagen levels. microRNA (miRNAs) is a small non-coding RNAs with a base number of 19-25 nucleotides. miRNA can inhibit target gene translation or degrade target gene RNA. miRNAs participate in a variety of cellular physiological processes and play important regulatory roles, such as differentiation, proliferation, apoptosis and metabolism [18]. miR-30c is one of miR-30 family members. Research shows that miR-30c expression is reduced in prostate cancer [19], which is similar to our research results. ROCK protein belongs to serine/threonine protein kinase, and it is divided into two protein subtypes of ROCK1 and ROCK2 [20]. Studies have shown that ROCK2 can promote the differentiation of epithelial cells and is relevant to the occurrence and development of tumors and many other diseases [21]. Yuan S et al. [22] pointed that ROCK2 expression in bladder cancer increased, and their research results supported ours. Urine albumin excretion rate (UAER) can be used to diagnose diabetic nephropathy, and has important clinical significance in judging DN classification and efficacy [23]. Twent-four hour urine protein quantification is one of the indispensable examinations for nephrotic patients, and urine protein content in nephrotic diseases can be increased markedly [24]. Cys-C can freely pass through the glomerulus and is completely reabsorbed in the proximal convoluted tubule. The renal tubule does not secrete Cys-C. Therefore, the concentration of Cys-C in blood is an ideal indicator to reflect the early glomerular filtration rate [25]. TGF-β1 can regulate cell growth and cellular morphology changes, participate in regulating cell proliferation, secretion and apoptosis through receptor signals, inhibit epithelial or endothelial cell growth, promote matrix production and inhibit matrix degradation, so a large amount of TGF-β1 produces excessive extracellular matrix, leading to fibrosis [26]. Collagen is an important component of basement membrane in the glomerular extracellular matrix, and its content accounts for more than 1/2. In the process of fibrosis, large amounts of

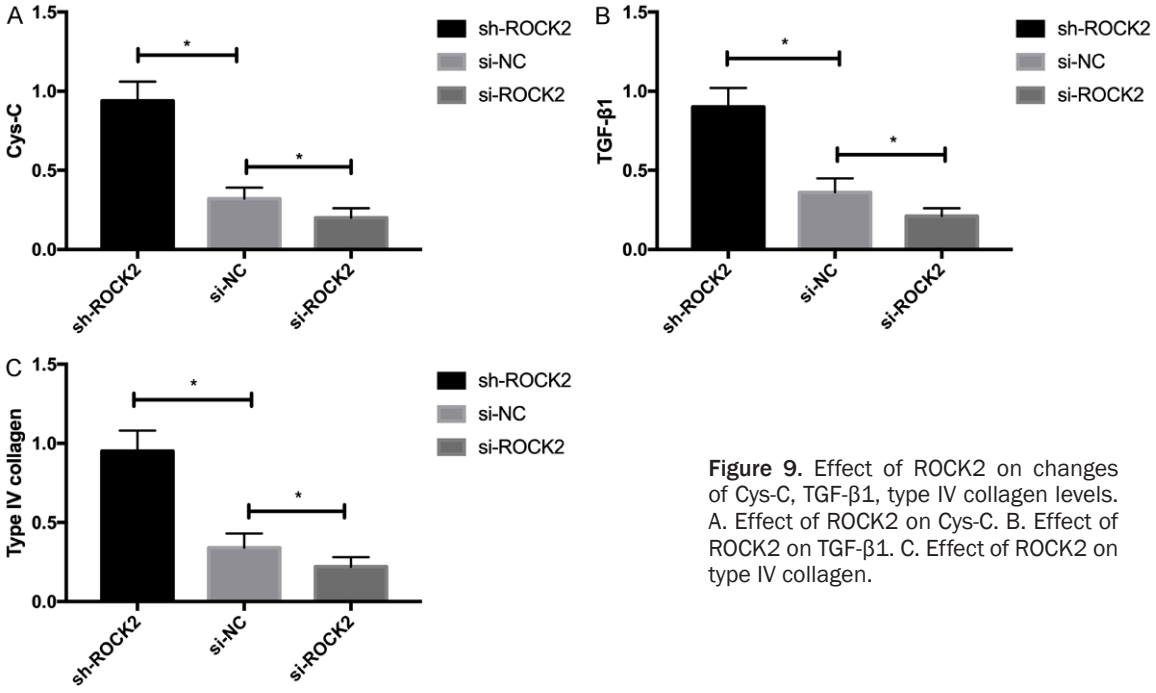

Figure 9. Effect of ROCK2 on changes of Cys-C, TGF-β1, type IV collagen levels. A. Effect of ROCK2 on Cys-C. B. Effect of ROCK2 on TGF-β1. C. Effect of ROCK2 on type IV collagen.

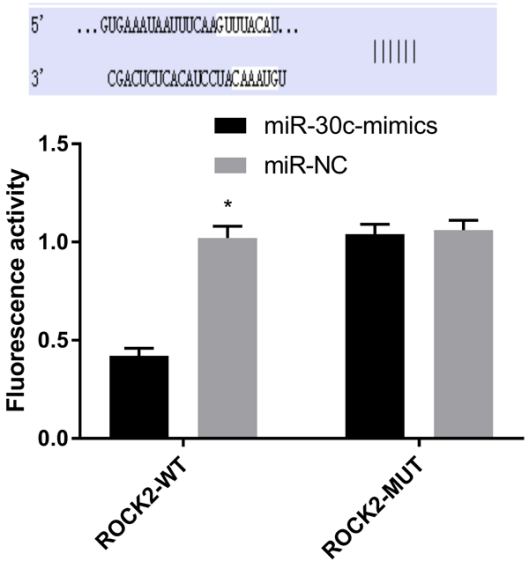

Figure 10. Binding sites of miR-30c and ROCK2 and the experimental results of dual-luciferase reporter enzyme; *means comparison with the fluorescence activity of ROCK2-WT transfected with miR-30c-mimics ($P<0.05$).

collagen deposition, especially the increase of type IV collagen, plays a vital part in the development of renal diseases [27]. Reviewing previous studies, we found that UAER, 24 h urine protein, Cys-C, TGF-β1 and collagen IV all showed an upward trend in nephropathy [28]. However, the author Ma T pointed out that miR-30c played an anti-cancer role in esophageal squamous cell carcinoma by targeting SNAI1 [29], while the mechanism by which miR-30c down-regulated ROCK2 affecting renal fibrosis was not clear. In the above-mentioned experiments, it was only preliminarily proved that miR-30c and ROCK2 affected UAER, 24 h urine protein, and the changes of Cys-C, TGF-β1 and type IV collagen levels. Hence, in order to verify our conjecture, we transfected cells. The results revealed that when miR-30c increased after transfection, UAER, 24 h urine protein and expression levels of Cys-C, TGF-β1, IV collagen decreased dramatically; when miR-30c decreased, UAER, 24 h urine protein and expression levels of Cys-C, TGF-β1, IV collagen increased dramatically; when ROCK2 increased, UAER, 24 h urine protein and expression levels of Cys-C, TGF-β1, IV collagen increased dramatically; ROCK2 decreased, UAER, 24 h urine protein and expression levels of Cys-C, TGF-β1, IV collagen decreased dramatically. These results further confirmed our above conjecture. Therefore, we can draw this conclusion: miR-30c can inhibit renal injury and fibrosis in DN by down-regulating ROCK2.

Due to limited conditions, these experiments still have some shortcomings. If the structure of rats is different from that of human body, it is not excluded that the results may be abnormal

when human body experiments are carried out. Moreover, due to the lack of basic experimental support, we still cannot fully understand the exact way ROCK2 regulates DN and miR-30c and related factors. In the end, we haven't verified the mechanism further, and we are not sure about the targeted relationship between the two. We will carry out experimental analysis for the above deficiencies as soon as possible to obtain more complete experimental results.

Conclusion

miR-30c with low expression in DN can promote the occurrence of renal injury and accelerate the process of renal fibrosis by increasing ROCK2 protein.

Disclosure of conflict of interest

None.

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