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Original Article

SiRNA-Cyp4a14 and diabetic nephropathy: silencing of Cyp4a14 by siRNA inhibits proliferation and fibrosis of mesangial cells

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Abstract: Diabetic nephropathy (DN) is one of the most common complications of diabetes, which increases mortality of diabetic patients. In recent years, many studies have confirmed that patients with diabetes are often accompanied by lipid metabolism disorders. Peroxisome proliferator-activated receptor (PPARs) is known to play pivotal roles in the regulation of insulin signaling, glucose and lipid metabolism. Cyp4a14 was confirmed to be the PPARα-target marker genes. We attempt to explore the biological role of Cyp4a14 in diabetic nephropathy. In our previous study, lncRNA microarray analysis showed us Cyp4a29-ps, Cyp4a14, Cyp4a12a and Cyp4a12b were the nearby mRNAs of lncRNA CYP4B1-PS1-001, we chose Cyp4a14 as our candidate. Renal cortical tissues were collected from db/db and db/m mice, and the mRNA expression of Cyp4a14 in diabetic tissue was significantly higher than that in normal tissue. And the expression of Cyp4a14 in mesangial cells cultured in high glucose was higher than that in low glucose by qualitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blot analysis. By Cell Counting Kit-8 (CCK8) and colony- forming ability, it is found that siRNA-Cyp4a14 treatment effectively suppressed the proliferation of mesangial cells. From our results, the protein expression of proliferating cell nuclear antigen (PCNA), Cyclin D1, Collagen I, and Fibronectin were all regulated dramatically in siRNA-Cyp4a14 group compared with the negative control group. Our data indicated that siRNA-Cyp4a14 inhibits proliferation and fibrosis of mesangial cells, which can be considered as a therapeutic target for diabetic nephropathy.

Keywords: Diabetic nephropathy, Cyp4a14, proliferation and fibrosis

Introduction

Diabetic nephropathy is the leading cause of end-stage renal disease, which is associated with the high morbidity and mortality, and the rapidly increasing prevalence become a health issue worldwide [1, 2]. Clinical treatment of diabetic nephropathy is mainly by improving glycemic control, control of blood lipids, strict control of blood pressure, anti-inflammatory treatment, anti-oxidation treatment, gene therapy and diet therapy of comprehensive treatment, but the treatment is difficult to achieve good results [3, 4]. The concept of the underlying pathophysiologic processes leading to diabetic nephropathy was considered as metabolic and hemodynamic alterations. However, fibrosis was the most fundamental and prominent feature of diabetic nephropathy [5, 6], but the exact pathogenic mechanisms and the molecular events of diabetic nephropathy remain incompletely understood.

Now, a number of studies indicated that oxidative stress, inflammation, and fibrosis are the key links in the progression of diabetic nephropathy [7, 8]. The cytochrome P450 proteins are involved in the synthesis of cholesterol, steroids, and other lipids, the proteins encoded by Cyp4a10 and Cyp4a14 hydroxylize fatty acids [9]. There is little report about Cyp4a14 gene. Cyp4a14 (cytochrome P450, family 4, subfamily a, polypeptide 14) is one of Cyp4a family in the mouse that involved in 20-HETE synthesis. The Cyp4a14 gene spans approximately 13 kb, and contains 12 exons, sequence similarity to

Table 1. The primers used in qRT-PCR

Target	Primer
Cyp4a14	Forward: 5'-TTTAGCCCTACAAGGTACTTGGA-3'
	Reverse: 5'-GCAGCCACTGCCTTCGTAA-3'
Cyp4a29-ps	Forward: 5'-TCTCCACTGGATATGGGGAAG-3'
	Reverse: 5'-GGTCAAGAATCGGTAGAGGCTA-3'
Cyp4a12a	Forward: 5'-TCCTCTAATGGCTGCAAGGC-3'
	Reverse: 5'-TGTCCAGGAAATCCAATCGCC-3'
Cyp4a12b	Forward: 5'-GGGGAGATCAGACCCAAAAGC-3'
	Reverse: 5'-ATTCGTCGGTGCTGAAACCAT-3'
PCNA	Forward: 5'-GGGAGCTTGGCAATGGGAACATTA-3'
	Reverse: 5'-TGTAGGAGACAGTGGAGTGGCTTT-3'
Cyclin D1	Forward: 5'-CTGGCCATGAACTACCTGGA-3'
	Reverse: 5'-ATCCGCCTCTGGCATTTTGG-3'
Collagen I	Forward: 5'-AAGGGTCCCTCTGGAGAACC-3'
	Reverse: 5'-TCTAGAGCCAGGGAGACCCA-3'
Fibronectin	Forward: 5'-AGACCATACCTGCCGAATGTAG-3'
	Reverse: 5'-GAGAGCTTCCTGTCCTGTAGAG-3'
β-actin	Forward: 5'-GTGACGTTGACATCCGTAAAGA-3'
	Reverse: 5'-GCCGGACTCATCGTACTCC-3'

the rat CYP4A2 gene sequence falls off 300 bp upstream from the start site [10]. Some study showed that the consequences of Cyp4a14 deletion were actually associated with an increase in renal 20-HETE production that could be attributed to the increased expression of a second Cyp4a enzyme (Cyp4a12) in close proximity to afferent arterioles [11]. Some study showed that Cyp4a14 was the PPARα-target marker gene, as we knew, PPARs played pivotal roles in the regulation of insulin signaling, glucose and lipid metabolism [12-14]. But the role of Cyp4a14 in diabetic nephropathy was unknown.

In our recent studies, we found that lncRNA CYP4B1-PS1-001 can effectively inhibit the proliferation and fibrosis of mouse mesangial cells (MMCs). And the microarrays showed Cyp4a14 was the nearby mRNA of lncRNA CYP4B1-PS1-001 [15], so we attempt to explore the effect of siRNA-Cyp4a14 silencing on proliferation and fibrosis in mouse mesangial cells (MMCs). This study demonstrated that Cyp4a14 plays an important role in diabetic nephropathy.

Materials and methods

Tissue samples from mice

The research was approved by the Animal Care and Ethical Committee of Nanjing Medical University.

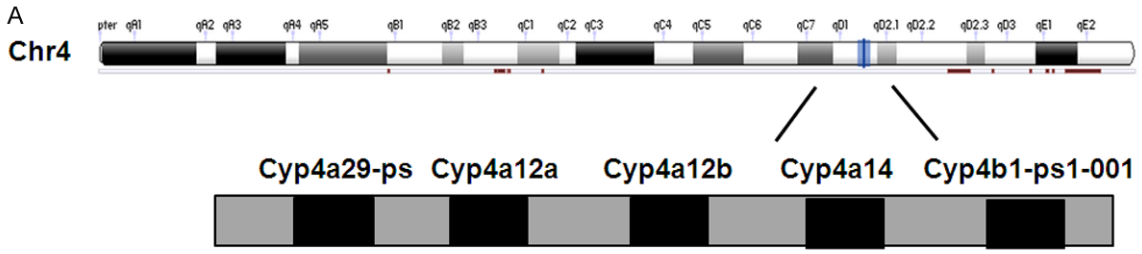
C57BL/KsJ background db/db mice were regarded as the model for type 2 diabetes. Three 9-week-old male db/db mice and three db/m mice as the controls were enrolled in this study for microarray analysis; all mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). Other twelve 8-week-old male db/db mice and twelve db/m mice as the controls were used for real-time quantitative PCR (qRT-PCR). Mice were killed at the age of 9 weeks in CO_2 asphyxiation. Kidneys were immediately excised and frozen in liquid nitrogen. Samples were stored at -70°C until required.

Cell culture

Mouse mesangial cells (MMCs) were obtained from the Shanghai Cell Bank, Chinese. Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 5 and 25 mmol/l glucose, respectively, to mimic normal and diabetic pathological physiological environments, 5% fetal bovine serum (FBS), and incubated in a humidified atmosphere at 37°C with 5% CO_2. HEK293T cells were cultured in DMEM with 5 mmol/l glucose and 10% fetal bovine serum, as previously described.

LncRNA and siRNA transfection

In our previous study, we get the plasmid pCDH CYP4B1-PS1-001 from a lentiviral system. The lentivirus CYP4B1-PS1-001 and an empty vector control were designated as Lv-CYP4B1-PS1-001 and Lv-Mock, respectively. We obtained virus supernatant co-transfection of pCDH-CYP4B1-PS1-001 or pCDH, packaging vector psPAX2 and envelope vector pMD 2.G from HEK293T cells. Then infected mouse mesangial cells (MMCs), after 48 hours, cells were collected and processed for qRT-PCR. Si 01 targeted to TACAGAGAAACAACTAATCTAGG, si 02 targeted to TTGTAGAAGTGATGATTTCTATG, and negative control targeted to AATTCTCCGAACGTGTCACGT were designed under the standard principles with GC ratio about 50% by RiboBio (RiboBio, Guangzhou, China). Mouse mesangial cells (MMCs) were seeded in antibiotic-free medium the day before transfection. The cells were transfected with 100 nmol/L of siRNA Cyp4a14 or negative control by using



Gene name	Origin of sequence
Cyp4a29-ps:	Chr4:115242084-54557
Cyp4a12a:	Chr4: 115299046-332815
Cyp4a12b:	Chr4: 115411624-39034
Cyp4a14:	Chr4:115486200-96159
Cyp4b1-ps1-001:	Chr4: 115543384-5069

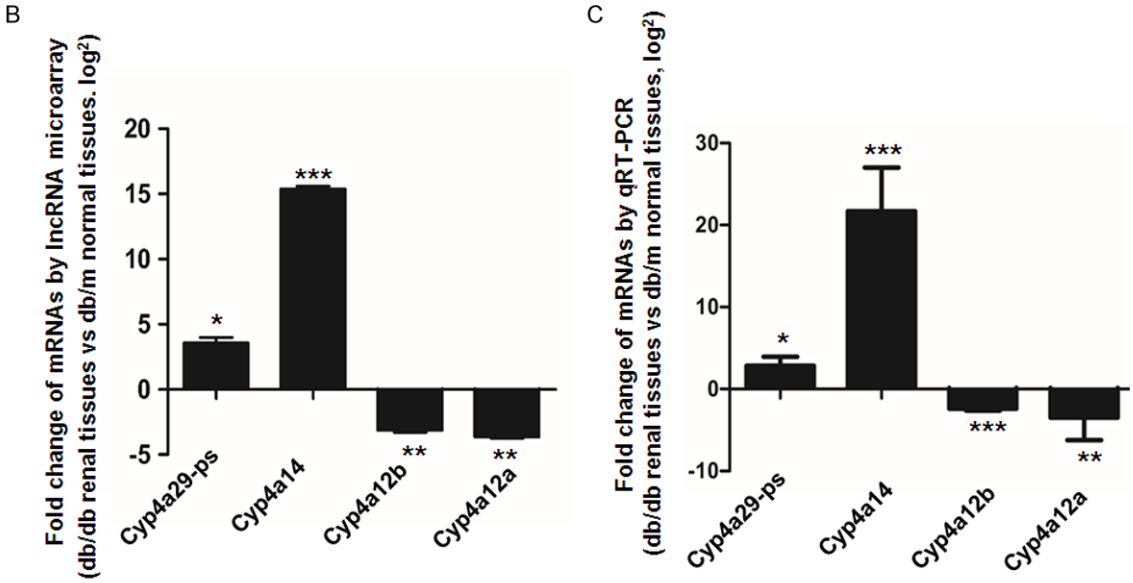


Figure 1. Differentially expressed mRNAs in renal tissues. (A) The position of Cyp4a14 in chromosome 4. (B) The expressions of four mRNAs (Cyp4a29-ps, Cyp4a14, Cyp4a12a, Cyp4a12b) by lncRNA microarray. (C) The results of A were verified in 16 pairs of db/db and db/m mice by qRT-PCR. Data are mean ± SD, $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ for Student's *t*-test.

Lipofectamine™ 2000 (Invitrogen, Shanghai, China) according to the instructions provided by the manufacturer. After 24 hours, the transfected cells were collected, the transfection efficiency was determined by qRT-PCR, and processed for western blot, proliferation.

Real-time quantitative PCR

RNA were isolated using Trizol reagent, were reverse transcribed into cDNA using a Prime Script™ RT Master Mix kit (Takara Biotechnology Co. Ltd., Dalian, China) according to the manufacturer's instructions. qRT-PCR was performed using SYBR Premix Ex Taq TM Kit (Takara) and ABI prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). Relative levels of gene expression were determined using β-actin as the control. qRT-PCR data were normalized to the expression of β-actin, and relative expressions were calculated using the $2^{-\Delta\Delta}$Ct method. The primers used in qRT-PCR are listed in **Table 1**.

Cell proliferation assay

Cell viability was assessed by Cell Counting Kit (CCK)-8 kit (Dojindo Molecular Technologies, Tokyo, Japan). 2×10^3 cells were seeded in each 96-well plate. Further incubated in an incubator (5% CO_2 at 37°C) for 0, 24, 48, 72, 96, and 120 h. CCK-8 reagent was added to each well at 1

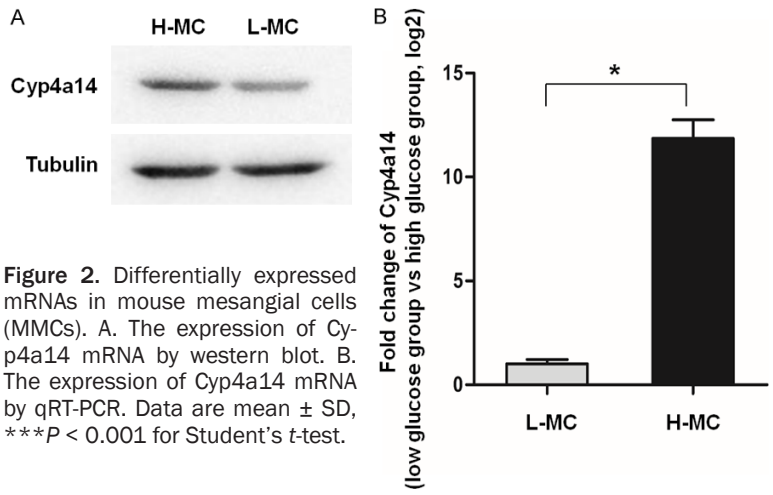


Figure 2. Differentially expressed mRNAs in mouse mesangial cells (MMCs). A. The expression of Cyp4a14 mRNA by western blot. B. The expression of Cyp4a14 mRNA by qRT-PCR. Data are mean ± SD, ***$P < 0.001$ for Student's *t*-test.

hour before the endpoint of incubation. The growth curves were detected by optical density (OD) 450 nm values in a microplate reader (iMark; Bio-Rad). Experiments were completed in triplicate. For the colony formation assay, two hundred transfected cells were seeded into a six-well plate and incubated in DMEM containing 5% FBS, being replaced every 4 days. Cells were cultured in the 37°C incubator for ten days until the majority of single colony contains more than 50 cells. Then, the cells were washed with PBS, fixed with paraformaldehyde, and stained with Giemsa. The colony formation was then manually counted. Three independent experiments were carried out. The formula for the colony formation ratio was as follows: Ratio = Numbers of Colony/Initiative Cells ×100%.

Western blot

Cultured or transfected cells were harvest and lysed in ice-cold radio immunoprecipitation assay buffer (RIPA) and 0.01% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, Missouri, USA), separated by 10% SDS-PAGE and transferred electrophoretically to polyvinylidene fluoride membranes (Millipore, Shanghai, China). The blots were blocked with 5% skim milk, followed by incubation with antibodies against Cyp4a14 (Santa), β-actin (Santa), PCNA (Abcam), Cyclin D1 (Abcam), Collagen I (Abcam), fibronectin (Abcam). Blots were then incubated with species-specific horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa), and visualized using Scion Image software (Scion Corporation, Frederick, MD, USA).

Statistical analysis

All data were presented as mean ± standard deviation (SD). Differences between the experimental groups were analyzed with the Student's t test. $P < 0.05$ was considered significant. All experiments were performed at least in triplicate unless otherwise stated.

Results

Expression of Cyp4a14 in diabetic tissues and normal tissues

In our previously study, microarray analysis showed that Cyp4a29-ps, Cyp4a14 were high in diabetic nephropathy, and Cyp4a12a, Cyp4a12b was low (**Figure 1B**). To verify the biological role of these mRNAs in diabetic nephropathy, we used QRT-PCR to detect the expression levels of mRNAs in mice renal tissues. We collected 16 db/db mice renal cortex tissues and normal db/m renal cortex tissues. As **Figure 1C** shows, the expression of them were the same with microarray analysis, we can see that Cyp4a14 expression level was higher in diabetic nephropathy tissues than that of normal tissues ($P < 0.01$); the results were consistent with the microarray data.

Cyp4a14 expression in mesangial cells

An obvious difference was presented in diabetic renal tissues and normal tissues. We then detected the mRNA expression and protein level of Cyp4a14 in mesangial cells by western blot and qRT-PCR. As shown in **Figure 2A**, Western blot show that Cyp4a14 mRNA expressions in high glucose MMCs were significantly higher than in low glucose. In addition, qRT-PCR displayed that the expression of Cyp4a14 protein level was the same with western blot (**Figure 2B**).

lncRNA CYP4B1-PS1-001 was regulated by Cyp4a14

The microarray showed Cyp4a14 was the nearby mRNA of CYP4B1-PS1-001, to investigate if CYP4B1-PS1-001 was under control of Cyp4a14, we overexpression of CYP4B1-PS1-001 by

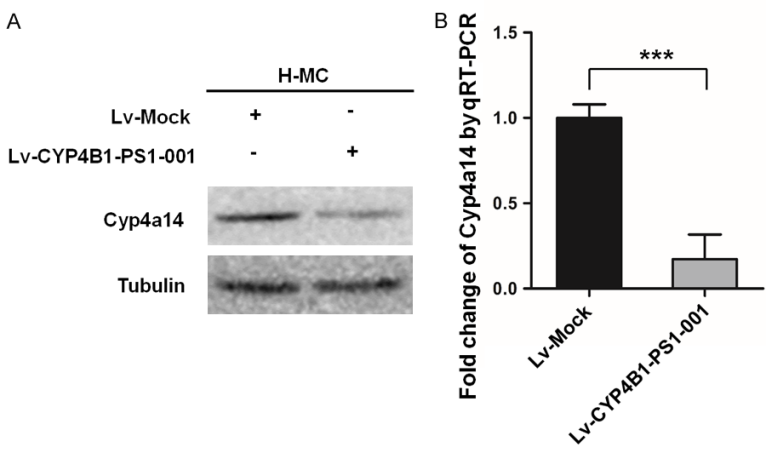


Figure 3. The expression of Cyp4a14 by overexpression of CYP4B1-PS1-001 in mouse mesangial cells (MMCs). A. Western blot indicated the expression of Cyp4a14 mRNA was low in MMCs by overexpression of CYP4B1-PS1-001. B. The expression of Cyp4a14 mRNA by overexpression of CYP4B1-PS1-001 by qRT-PCR. Data are mean ± SD. ***$P < 0.001$ for Student's *t*-test.

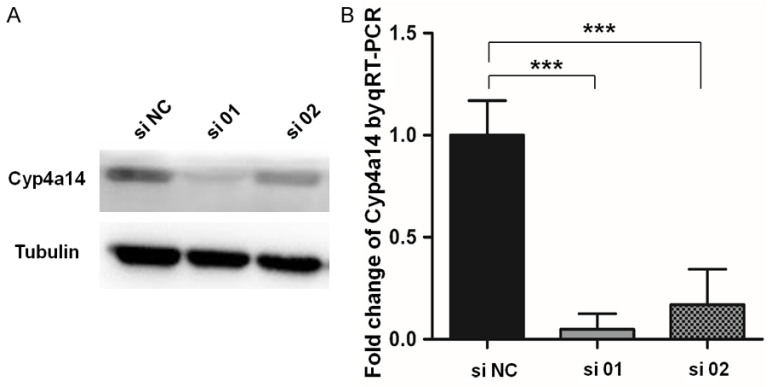


Figure 4. The expression of Cyp4a14 by siRNA in mousemesangial cells (MMCs). A. The expression of Cyp4a14 were lower in MMCs of transfected with si 01 and si 02 than si NC by western blot. B. The expression of Cyp4a14 in MMCs treated as in A was detected by qRT-PCR. Data are mean ± SD. ***$P < 0.001$ for Student's *t*-test.

retrovirus infection in MMCs, then use Western blot and qRT-PCR to detect the expression of Cyp4a14, the results showed that the expression of Cyp4a14 was significantly increased in mouse mesangial cells (MMCs) transduced with Lv-CYP4B1-PS1-001 compared with Lv-Mock (**Figure 3**).

Silencing of Cyp4a14 by siRNA in MMCs

To investigate the role of Cyp4a14 in diabetic nephropathy progression, the siRNAs targeted on Cyp4a14 were firstly transfected to mouse mesangial cells (MMCs) by Lipofectamine™ 2000. Western blot indicated successful infection of siRNAs in MMCs and siRNAs can significantly reduce the expression of Cyp4a14 (**Figure 4A**). Meanwhile, the results of qRT-PCR confirmed the same results with Western blot (**Figure 4B**).

siRNA-Cyp4a14 inhibited the proliferation and fibrosis of mesangial cells

Cyp4a14 mRNA was interfered in mesangial cells as previously described. Western blot was employed to identify the interference efficient. Western blot showed that Cyp4a14 protein level was declined notably in siRNA Cyp4a14 group in comparison with the negative control group in mesangial cells and the high glucose of mouse mesangial cells (MMCs). In addition to explore the effect of siRNA Cyp4a14 on the proliferation of mesangial cells, CCK8 assay was employed to identify the cell proliferation. The results from Western blots also showed that knockdown Cyp4a14 in MMCs under high glucose conditions decreased the levels of PCNA and fibronectin (**Figure 5A**). Consistent with western blot results, transcripts of Cyclin D1 and Collagen I detected by qRT-PCR were increased in MMCs under high glucose conditions and si NC (**Figure 5B**), downregulated after si 01 and si 02 transduction. As shown in **Figure 6**, proliferation of siRNA-Cyp4a14 group was significantly depressed compared with negative control group at 5 days. These data indicated that knockdown of Cyp4a14 participates in MMCs proliferation during the progress of diabetic nephropathy.

Discussion

Diabetic nephropathy is the most common single cause of end-stage renal disease and one of the most significant long-term complications

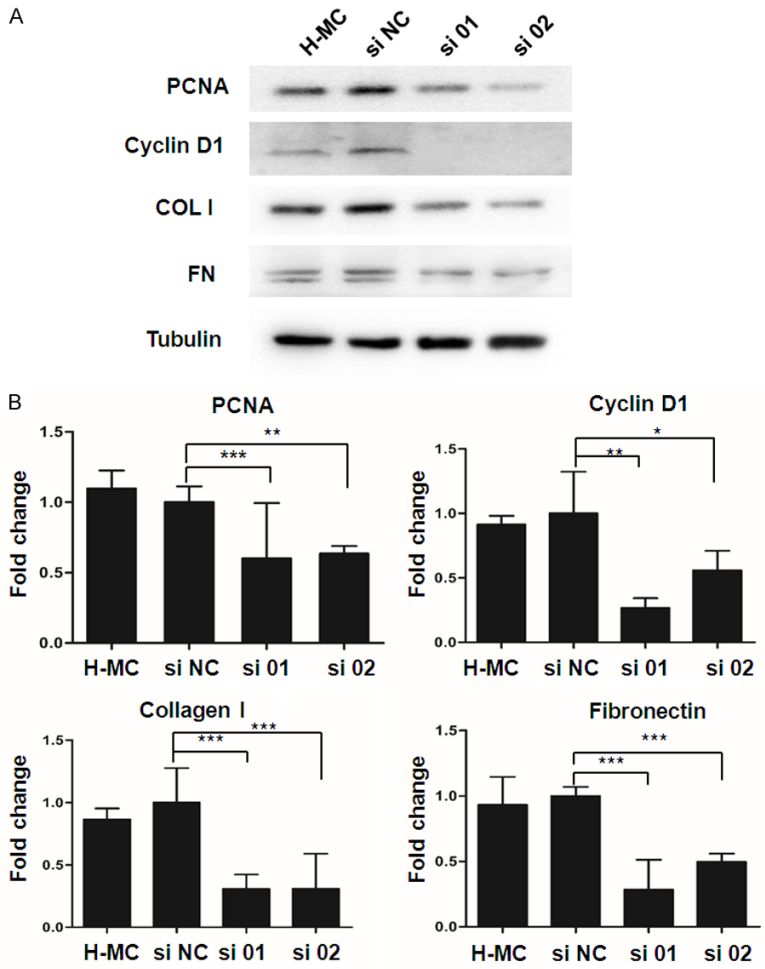


Figure 5. Expression of PCNA, Cyclin D1, Collagen I and fibronectin as the indicators of proliferation and fibrosis in mouse mesangial cells (MMCs). A. Western blots to measure the expression of PCNA (PCNA), Cyclin D1 (Cyclin D1), Collagen I (COL I) and fibronectin (FN) in MMCs under high glucose (H-MC, 25 mmol) conditions without transduction and in MMCs transduced with si 01, si 02 or si NC under high glucose conditions. B. qRT-PCR for the mRNA levels of PCNA, Cyclin D1, Collagen I and fibronectin in MMCs treated as in A. Data are mean ± SD. ***P < 0.001 for Student's *t*-test.

associated with diabetes. Epidemiological studies show increasing prevalence of diabetic nephropathy [16, 17]. However, the treatment of diabetic nephropathy has not been elucidated yet, so it is important to understand the molecular mechanism underlying diabetic nephropathy. Diabetic nephropathy is characterized by extracellular matrix (ECM) accumulation, glomerulosclerosis, epithelial-to-mesenchymal transition (EMT) of renal tubular cells and tubular interstitial fibrosis, which ultimately lead to a progressive loss of renal function, extracellular matrix deposit was regarded as early event of progressive fibrosis [18, 19], various factors such as hemodynamic changes, metabolic pathways, excessive glucose influx activates cellular signaling pathways have been shown to be involved in the pathogenesis of diabetic nephropathy [20], but the pathogenesis of diabetic nephropathy is a complex process and it is not yet fully understood. Many new technologies have identified that most of the genome is transcribed, producing a heterogeneous population of RNAs which do not encode for proteins (ncRNAs), but the mechanisms and functions of different classes of ncRNA still remain unknown [21]. The main classes of ncRNAs, such as messenger RNA, transfer RNA, microRNA and long noncoding RNAs, play essential roles in the regulation of gene expression and mammalian development and disease processes [22]. Many new technologies have identified that most of the genome is transcribed, producing a heterogeneous population of RNAs which do not encode for proteins (ncRNAs), but the mechanisms and functions of different classes of ncRNA still remain unknown [21]. The main classes of ncRNAs, such as messenger RNA, transfer RNA, microRNA and long noncoding RNAs, play essential roles in the regulation of gene expression and mammalian development and disease processes [22]. Many studies demonstrated that miRNAs extensively implicated in the pathogenesis of diabetic nephropathy, miR-192 was downregulated by a locked nucleic acid-modified anti-miRNA in the renal cortex to improve renal fibrosis symptoms in streptozotocin-treated diabetic mice, miR-21 inhibitor the progression of renal fibrosis and ameliorated renal structure and function in diabetic nephropathy [23]. However, studies on lncRNAs have not gained much attention. It is reported that overexpression of lncRNA *PVT1* increases cell proliferation and inhibits apop-

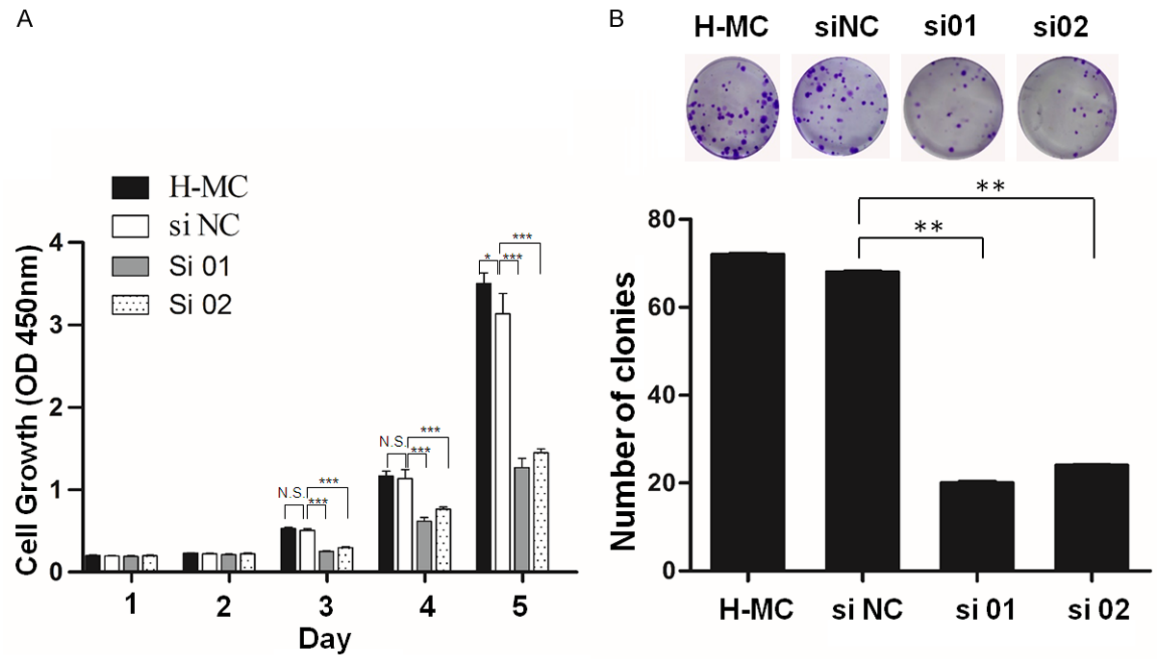


Figure 6. Silencing of Cyp4a14 inhibited mouse mesangial cells (MMCs) proliferation and colony formation ability. A. The growth curves of mouse mesangial cellsdetermined by CCK-8 assay ($P < 0.01$). B. Colony formation assays showed that the siRNA-Cyp4a14 inhibited the ability of colony formation compared with the control group in mouse mesangial cells (*$P < 0.001$).

tosis, and *PVT1* may mediate the development and progression of diabetic nephropathy through mechanisms involving ECM accumulation. lncRNA MIAT (myocardial infarction-associated transcript) is significantly lower in diabetic rats and patients, MIAT can bind to Nrf2 protein, knock down of MIAT reduced cell viability, whereas this inhibitory action was abrogated by Nrf2 overexpression [24].

Cytochrome P450 family 4 (CYP4) proteins are important for chemical defense, they metabolize fatty acids, eicosanoids, and vitamin D. The production of kidney CYP450 arachidonic acid metabolites alter renal hemodynamics, tubular transport processes, and various hypertension, which contributes to the abnormalities in renal function [25]. The CYP4A subfamily is cytochrome P450 (P450) fatty acid hydroxylases that catalyze the ω-hydroxylation of medium and long chain fatty acids and prostaglandins. Inhibition of CYP4A in mice reduces insulin resistance, steatosis, apoptosis, and hepatic ER stress. Strategies to reduce levels or activity of CYP4A proteins in liver might be developed for treatment of patients with type 2 diabetes [26]. Cyp4a14 was PPARα-target marker genes. The peroxisome proliferator activated receptor-α (PPAR-α) plays a pivotal role in the regulation of lipid metabolism and fatty acid oxidation, and is beneficial in protecting against metabolic disorders associated with type-II diabetes and obesity [13, 27]. These published reports implicate there should be some relationships between Cyp4a14 and the development of diabetic nephropathy.

In this study, microarray analysis was used to screen for abnormally expressed lncRNAs and nearby mRNAs in renal tissues of diabetic nephropathy and normal mice, and the GO analysis showed most mRNAs are mainly involved in the pathogenesis of diabetic nephropathy, including immune processes, steroid dehydrogenase activity, and protein binding. The data showed four nearby mRNAs of lncRNA CYP4B1-PS1-001, two nearby upregulated mRNAs Cyp4a29-ps, Cyp4a14 and two downregulated mRNAs Cyp4a12a and Cyp4a12b, with differential expression by > 2-fold. Therefore, we studied the role of Cyp4a14 in mouse mesangial cells, other mRNAs should be deep explored in the later research. Firstly, we found that Cyp4a14 expressed obviously higher in diabetic renal tissues than in normal tissues. Cyp4a14 was regulated by lncRNA CYP4B1-PS1-001, but

the regulatory mechanism should be sought out in our later studies. Diabetic nephropathy is a complex multistep process including cell proliferation and fibrosis. In our study, the results indicated that siRNA Cyp4a14 showed obvious inhibition of proliferation and fibrosis in mesangial cells.

To conclude, we demonstrated that siRNA Cyp4a14 can obviously inhibit the proliferation and fibrosis of mesangial cells. Our results may also be relevant for human NSCLC carcinoma therapy.

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Disclosure of conflict of interest

None.

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