**MicroRNA-23b Targets Ras GTPase-Activating Protein SH3 Domain-Binding Protein 2 to Alleviate Fibrosis and Albuminuria in Diabetic Nephropathy**

Binghai Zhao,* Hongzhi Li,* Jieting Liu,* Pengfei Han,† Chunlei Zhang,* He Bai,* Xiaohuan Yuan,* Xiaoli Wang,† Li Li,* Hongchuang Ma,* Xiudong Jin,* and Yanhui Chu*

*Heilongjiang Key Laboratory of Anti-fibrosis Biotherapy, Medical Research Center, and †Clinical Laboratory of Hong Qi Hospital, Mudanjiang Medical University, Heilongjiang, People’s Republic of China

**ABSTRACT**

Diabetic nephropathy (DN) is a frequent and severe complication of diabetes that is structurally characterized by glomerular basement membrane thickening, extracellular matrix accumulation, and destabilization of podocyte foot processes. MicroRNAs (miRNAs) are dysregulated in DN, but identification of the specific miRs involved remains incomplete. Here, we confirm that the peripheral blood from patients with diabetes and the kidneys of animals with type 1 or 2 diabetes have low levels of miR-23b compared with those of their non diabetic counterparts. Furthermore, exposure to high glucose downregulated miR-23b in cultured kidney cells. In contrast, renal expression of Ras GTPase-activating protein SH3 domain-binding protein 2 (G3BP2), a putative miR-23b target, increased in DN. In vitro, overexpression of miR-23b decreased, and inhibition of miR-23b increased, G3BP2 expression levels. Bioinformatics analysis also revealed p53 binding sites in the miR-23b promoter; in vitro inhibition of p53 or the upstream p38 mitogen-activated protein kinase (p38MAPK) upregulated miR-23b expression in high-glucose conditions. In turn, inhibition of G3BP2 or overexpression of miR-23b downregulated p53 and p38MAPK expression in high-glucose conditions. In vivo, overexpression of miR-23b or inhibition of p53 in db/db mice reversed hyperalbuminuria and kidney fibrosis, whereas miR-23b antagomir treatment promoted renal fibrosis and increased albuminuria in wild-type mice. These data suggest that hyperglycemia regulates pathogenic processes in DN through an miR-23b/G3BP2 feedback circuit involving p38MAPK and p53. In conclusion, these results reveal a role for miR-23b in DN and indicate a novel potential therapeutic target.


Received March 20, 2015. Accepted December 16, 2015.

Published online ahead of print. Publication date available at www.jasn.org.

Correspondence: Prof. Yanhui Chu or Prof. Xiudong Jin, Heilongjiang Key Laboratory of Anti-fibrosis Biotherapy, Mudanjiang Medical University, 3 Tongxiang Street, Mudanjiang, 157001, China. Email: yanhui_chu@163.com or mdjmujin@163.com

Copyright © 2016 by the American Society of Nephrology

---

Diabetic nephropathy (DN) is a major complication of diabetes and remains a leading cause of chronic renal failure in individuals with both type 1 and type 2 diabetes. The major structural characteristics of DN include proteinuria, glomerular basement membrane (GBM) thickening, and mesangial expansion due to accumulation of extracellular matrix (ECM) proteins.1 Despite the identification of multiple pathogenic mechanisms in a complex problem like DN, limited success has been achieved in its medical treatment. Currently there are no specific medical treatments to prevent fibrosis in DN.2

Ras GTPase-activating protein SH3 domain-binding protein 2 (G3BP2), a member of the G3BP family of the Ras network, is overexpressed in human cancers and involved in a variety of growth-related signaling pathways that are of importance in carcinogenesis and metastasis.3 G3BP2 acts through NF-κB, Ras signaling, and the mitogen-activated protein kinase pathways.4 Within the mitogen-activated protein kinase family, p38 kinase (p38) and c-Jun N-terminal kinase are activated in response to hyperglycemia. Increased activation of p38 has been identified as a fundamental mechanism responsible for renal dysfunction in diabetes.5,6 Oxidative stress and inflammatory processes have been demonstrated to be important contributors in the pathogenesis of several chronic diabetes complications, including DN.7,8 As a result of such inflammatory processes, increased ECM protein production—a characteristic pathologic component—occurs in all chronic diabetes complications, such as DN.9–10 G3BP2 is also a component of stress granules (SGs) and the processing body (P-body). Oxidative stress and inflammatory processes further promote...
P-body and SG formation.\textsuperscript{11,12} However, whether G3BP2 plays a role in DN and how it is regulated in this context is not clear.

Since the discovery of microRNAs (miRNAs), accumulating evidence indicates that they play an important role in various diseases, including diabetes and kidney dysfunction.\textsuperscript{13} Furthermore, a podocyte-specific conditional knockout of Dicer, an essential enzyme involved in the processing of miRNAs, resulted in progressive renal glomerular and tubular damage in mice.\textsuperscript{14,15} Several miRNAs, including miR-192/216a/217,\textsuperscript{16} miR-200a,\textsuperscript{17} and miR-29a/b/c,\textsuperscript{18} are involved in DN. During a search using an open-source bioinformatics analysis, we identified miR-23b as a candidate miRNA targeting G3BP2. Additionally, miR-23b has been shown to be an important regulator of the innate immune response in cancer and in several inflammatory processes.\textsuperscript{19} In this study, we have shown that miR-23b is a commonly downregulated miRNA in the serum of patients with DN, in the kidneys of mouse models of type 1 and 2 diabetes, and in specific kidney cells exposed to high glucose. We also detected G3BP2 upregulation in cells exposed to high glucose and in the kidneys of mice with both type 1 and type 2 diabetes. We further examined whether miR-23b may have a protective role in the pathogenesis of DN through alteration of G3BP2 signaling.

Measurement of miR-23b levels in the serum of patients with diabetes showed miR-23b is downregulated in these patients, with or without nephropathy, compared with the healthy controls (Figure 1A). We then demonstrated by quantitative PCR (qPCR) a similar downregulation of miR-23b present in kidney tissues of streptozocin-induced diabetic and db/db mutant mice (Figure 1, B and C, Supplemental Figure 1, A–C) and in cultured kidney cells (Figure 1, D–F) exposed to high glucose. However, miR-23a/c had different expression compared with miR-23b in the serum of patients with diabetes versus that of diabetic mice (Supplemental Figure 1, D–E, and miR-23c data not shown). The expression level of miR-23a was not altered when miR-23b increased or decreased (Supplemental Figure 1, F, G, I, and J). We further found that urine protein levels in type 2 DN display an inverse correlation with miR-23b (Figure 1G). A minimum threshold level of expression must be reached for miRNAs to repress their target mRNAs, and the abundance of miRNAs in the miRNAome of a specific cell or tissue may be more important for their functions.\textsuperscript{20} To explore such a notion, we examined miR-23b levels in multiple tissues, including the kidneys, and found that miR-23b was highly expressed in mouse heart and kidneys compared with other tissues (Figure 1H, Supplemental Table 1).\textsuperscript{14}

We used bioinformatics analyses (Targetscan, miRanda, StarBase, and mirDB) to narrow our focus onto G3BP2, a target of miR-23b (Supplemental Figures 1N and 2). We performed qPCR to establish whether G3BP2 expression was altered in DN. G3bp2 mRNA levels were upregulated in cells exposed to high glucose and in the kidney tissues of db/db mice compared with controls (Figure 1, I and J). Because miRNAs are negative regulators of their targets, among the normal mouse tissues measured we found lower levels of G3bp2 mRNA in the kidney and heart. However, relatively higher levels of G3bp2 mRNA were seen in tissues with low miR-23b expression (e.g., testis, Figure 2A). To establish whether G3bp2 was a target of miR-23b in vivo we transfected HK-2 cells with a miR-23b mimic. Transfection efficiencies assessed by measuring miR-23b expression showed a greater than tenfold increase compared with scrambled miRNA transfection (Supplemental Figure 1F). After transfection, G3BP2 protein and mRNA levels were decreased in HK-2 cells (Figure 2B, Supplemental Figure 1H). Similarly, miR-23b antagonist transfection was associated with a greater than fivefold decrease in cellular miR-23b levels (Supplemental Figure 1I). In addition, normal G3BP2 protein levels were increased by inhibition of miR-23b (Figure 2C). We also carried out luciferase assays, which confirmed that miR-23b overexpression significantly repressed G3BP2 3′-UTR luciferase activity. However, we saw no such repression when we performed similar experiments using a mutated version of its 3′-UTR (Figure 2D). These data identified miR-23b binding to the 3′-UTR of its putative target G3BP2 mRNA.

Several recent studies have identified miRNAs directly regulated by key transcription factors that define and drive cellular differentiation.\textsuperscript{21–26} Another report indicated miR-23b promoters were responsive to p53.\textsuperscript{27} Therefore, we explored whether miR-23b expression in DN is controlled by known transcription factors, such as p53. We used bioinformatics analyses (PROMO) that revealed 29 consensus p53 binding sites in the miR-23b promoter (Supplemental Figure 1L). We further confirmed that p53 was increased in HK-2 cells exposed to high glucose and kidney tissues of diabetic animals (Figures 3, A–C, and 4H).\textsuperscript{28,29} MiR-23b expression further increased after p53 small interfering RNA (siRNA) transfection into HK-2 cells treated with high glucose, but not after transfection of a scrambled siRNA (Figure 3D). Because p53 is a downstream target of p38 mitogen-activated protein kinase (p38MAPK) signaling, we examined p53 levels after incubation with p38MAPK inhibitor SB203580, and found p53 downregulation in HK-2 cells in spite of the high-glucose treatment (Figure 3A). However, such treatment-induced signal inhibition was associated with increased miR-23b expression levels (Figure 3E). These data indicate that p38MAPK regulates miR-23b expression possibly via p53. In an attempt to detect a relationship between G3BP2 and p38MAPK, we transfected G3BP2 siRNA into HK-2 cells under high-glucose conditions, and demonstrated a decrease in p38MAPK levels (Supplemental Figure 1M). Furthermore, to potentially discover a cause-and-effect relationship, we incubated HK-2 cells in high glucose with p38 inhibitor SB203580 and examined G3BP2 mRNA and protein levels. There was a small, nonsignificant decrease in G3BP2 mRNA and protein levels in these experiments (Figures 2C and 3G,
Supplemental Figure 1M). These data support the notion that G3BP2 is located upstream of p38MAPK. We also confirmed that p53 expression was decreased and miR-23b was upregulated by transfection of G3BP2 siRNA into HK-2 cells in high glucose (Figure 3, C and F). We also established that overexpression of miR-23b could decrease p53 expression and
G3BP2 expression, respectively (Supplemental Figure 3, A and B). Interestingly, the body weight and abdominal fat weight of db/db mice were significantly decreased after miR-23b agomir and G3bp2 siRNA injection (Figure 4, A and B), but this alteration was not related to food intake (Figure 4C). Hence, miR-23b and G3bp2 siRNA may have additional effects on obesity in db/db mice. Although serum glucose and insulin showed no significant difference from controls in the miR-23b agomir- and G3bp2 siRNA-treated mice (Figure 4, D and E), we discovered that miR-23b agomir and G3bp2 siRNA lowered the insulin resistance index and increased insulin sensitivity (Figure 4, F and G). This may be one of the reasons for the reduced body weight; however, the exact mechanism for such changes needs to be explored further. We also discovered that p53 and p38MAPK were decreased after miR-23b agomir and G3bp2 siRNA delivery (Figure 4, H and I). These data further indicate the existence of a miR-23b/G3BP2 feedback circuitry in vivo. We also found that kidney weight was increased significantly in miR-23b agomir- or G3bp2 siRNA-treated mice compared with db/db mice and miRNA negative control (miRNEG)- or siRNA negative control (siRNEG)-injected mice (Supplemental Figure 3, E and F). Importantly, we found miR-23b agomir and G3bp2 siRNA injection decreased microalbuminuria and the albumin-to-creatinine ratio (Figure 4, J and K). Increased microalbuminuria is always due to collapse of the podocyte foot processes and GBM thickening, so we used electron microscopy to examine whether miR-23b agomir and G3bp2 siRNA could reverse such abnormalities. Fortunately, flattening of the podocyte foot processes in db/db mice was corrected after miR-23b agomir and G3bp2 siRNA injections (Figure 4L). Diabetes-induced GBM thickening was also reversed by miR-23b agomir and G3bp2 siRNA injections (Figure 4M, Supplemental Figure 3G).

One characteristic of DN is fibrosis, including mesangial hypertrophy and ECM deposition. To examine if miR-23b and G3bp2 siRNA could reverse
kidney fibrosis, first we identified that the serum miR-23b expression level is negatively correlated to TGF-β1 production, but that of miR-23a is not, in patients with diabetic nephropathy (Supplemental Figure 3, C and D). We also performed immunofluorescence for fibronectin (FN), which showed increased FN production in HK-2 cells exposed to high glucose (Figure 2E). That alteration was inhibited by miR-23b mimic and G3bp2 siRNA transfection (Figure 2E). Then, we found that miR-23b agomir or G3bp2 siRNA injection could reverse glomerular fibrosis as evidenced by periodic acid–Schiff (PAS), FN, fibroblast-specific protein 1, smooth muscle actin (α-SMA), and collagen I

Figure 3. miR-23b/G3BP2 feedback circuitry. (A–C) Quantification of P53 mRNA expression in HK-2 cells treated with HG, p38MAPK inhibitor SB203580, miR-23b(+), and G3BP2 siRNA (G3BP2 siR1, 2, 3). (D–F) Quantification of miR-23b expression levels in HK-2 cells exposed to HG, p53 siRNA, SB203580, and G3BP2 siRNA. (G) Quantification of G3BP2 mRNA expression in HK-2 cells incubated with SB203580 exposed to HG. (H) Western blot analyses for p38, phosphorylated p38 (p-p38), p53, and phosphorylated p53 (p-p53) from HG, NG, miR-23b agomir, miRNEG, and OSM (right panels showing quantification). (I) A schematic model of potential regulation and function of miR-23b in diabetic nephropathy. Data are expressed as the mean±SEM. *P<0.05; **P<0.01; ***P<0.001.
Figure 4. Intravenous injection of miR-23b and G3BP2 siRNA alleviate albuminuria in diabetes. (A–C) Quantification of body weight (BW), abdominal fat, and food consumption of db/db mice with or without miR-23b agomir [miR-23b(+) or G3bp2 siRNA (G3BP2siR) injection weekly for 1 month (for P values [see below], * indicates miR-23b versus db/db, and # indicates G3bp2 siRNA versus db/db, n=5). (D–G) Quantification of serum glucose and insulin levels, insulin resistance index, and insulin sensitivity index from db/db mice and miR-23b agomir or G3BP2 siRNA injection.
and IV staining in db/db mice (Figure 5A, Supplemen
tal Figure 3I). Subsequently, we analyzed additional kidney
fibrosis-related transcripts by qPCR, and discovered increased mRNA expres-
sion of collagen1α (I), collagen4α (IV), Timp1, Tgf-β1, Acta2, Papi1, and Fn in
db/db mice. However, these upregulated fibrosis-related genes were sup-
pressed by mir-23b agomir or G3bp2 siRNA in-
jection (Figure 5B).

Overexpression of p53 in normal mice resulted in kidney dysfunc-
tion, including small kidneys, proteinuria, and fibro-
sis. To further establish the miR-23b/G3BP2 feedback cir-
cuit, we also in-
jected p53 siRNA (2.5 mg/kg) into db/db mice for 1 month. Delivery efficiencies
showed an almost threefold decrease in kidney p53 expression (Supplemen-
tal Figure 4A). Interestingly, when p53 ex-
pression was decreased, mir-23b expression was significantly increased but
G3bp2 was reduced (Supplemental Figure 4, B and C). This strongly indi-
cates that mir-23b is regulated by G3BP2, P38MAPK, and the P53 cascade
through a miR-23b/G3BP2 feedback cir-
cuit. We also found that downregu-
lation of p53 resulted in lower proteinuria
compared with db/db mice and siRNA-
treated mice (Supplemental Figure 4, D
and E). Consistent with the proteinuria
alteration, inhibition of p53 in db/db mice led to a reversal of kidney fibrosis
as evidenced by a histologic analysis in-
cluding hematoxylin and eosin, PAS,
α-SMA, and transmission electron mi-
croscopy (Supplemental Figure 4, F–H).

Because mir-23b was downregu-
lated in diabetes, we explored whether in-
hibition of mir-23b could accelerate
kidney fibrosis and urine production in
wild-type mice. Therefore, normal
(nondiabetic) mice were treated intrave-
ously with 80 mg/kg mir-23b antag-
gomir or miRNEG antagonist for 3
consecutive days and were euthanized at
the end of 1 month. miRNA inhibition
ef
ciencies showed an approximately sixfold decrease (Supplemental Figure 5A).
Such downregulation was associ-
ated with an increase in G3bp2 mRNA
expression (Supplemental Figure 5A).
Furthermore, PAS staining and histo-
logic analysis for FN, α-SMA, fibroblast-
specific protein 1, and collagen 1/II
showed increased glomerular and renal
tubular fibrosis in mice receiving mirR-
23b antagonist compared with controls
(Figure 6, A–C, Supplemental Figure 5B).
A similar alteration was also de-
tected in HK-2 cells exposed to low glu-
cose and transfected with mir-23b
antagonist (Figure 2E). We also ana-
lyzed fibrosis-related transcripts by qPCR.
In the mir-23b antagonist-treated
mice, the kidneys showed increased
mRNA expression of collagen1α (I),
collagen4α (IV), Timp1, Tgf-β1, Acta2,
Pai1, and Fn relative to controls and
miRNEG mice (Figure 6D). In keeping with
these findings, electron micros-
copy showed mesangial expansion in
mir-23b antagonist-treated mice, but
not in the mice receiving miRNEG
injection (Figure 6E). We further ob-
served that inhibition of mir-23b re-
sults in flattening of podocyte foot
processes, GBM thickening, and pro-
teinuria (Figure 6, E–I, Supplemental
Figure 5B). Interestingly, we further
confirmed that demonstration that inhi-
bration of mir-23b could activate p38MAPK and p53
(Figure 6, J and K). Such alteration
again strongly indicated the existence of
a miR-23b/G3BP2 feedback circuitry
in vivo.

This study showed a protective effect of
miR-23b in DN and identified that
G3BP2 is a target of miR-23b. Diabetic
kidneys showed increased ECM protein
production and G3bp2 mRNA levels.
miR-23b agonist injection inhibited
such processes in DN and also reduced
proteinuria in vivo, possibly by inhib-
it ing podocyte dysfunction. Notably,
inhibition of mir-23b in normal mice
resulted in increased kidney fibrosis,
podocyte collapse, and proteinuria. Ad-
ditionally, we have shown that hypergly-
cemia regulates this process through a
miR-23b/G3BP2 feedback circuitry.

In diabetes, overproduction of super-
oxides by the mitochondrial electron
transport chain as a result of hypergly-
cemia increases the proton gradient and
causes DNA damage and activation of
several nuclear proteins leading to the
activation of transcription factors and
gene expression. miRNAs provide an-
other level of transcriptional regulation.
By binding to the 3’-UTR of specific
mRNAs, miRNAs cause their degrada-
tion or translational repression. Although
miR-23b regulates cell metab-
olism, cancer development, and autoim-
une pathogenesis, a role for
miR-23b in the pathogenesis of DN
was previously unknown. In this study
we have shown that miR-23b suppresses
development of DN. miR-23b has been
shown to target glutaminase and regu-
late glutamine metabolism. The
miRNA also regulates inflammation
by targeting TGF-β-activated kinase
1 (MAP3K7) binding protein 2/3 and
inhibitor of nuclear factor κ-β kinase
subunit-α. Here, we identified G3BP2 as
an additional target of mir-23b that acts
in signaling cascades downstream of
hyperglycemia. Although G3BP2 has been
shown to be crucial for formation of SGs and P-bodies, its involvement in DN was previously unknown.4,38,39 Liu et al. reported that mRNAs that are targeted for translational repression by endogenous or exogenous miRNAs become concentrated in P-bodies in a miRNA-dependent manner.40 In this study, we have confirmed that downregulation of G3BP2 by miR-23b could decrease kidney fibrosis and alleviate proteinuria in DN. Additionally, we found evidence to support miRNA involvement in P-body-induced translational repression of proteins. Thus, these findings raise a new question—whether SGs and P-bodies play a role in diabetic complications—that needs to be resolved in the future. One of the challenges regarding miRNA-based therapy is that one mRNA can be regulated by multiple miRNAs, and one miRNA can regulate multiple mRNAs. Hence, miR-23b may possess additional targets that need further characterization. Nevertheless, the data from this study are novel and taken together show that miR-23b and G3BP2 could be potential targets to treat DN.

p38MAPK signaling plays a key role in DN.41 p38MAPK signaling is known to promote inflammatory and profibrotic responses and has been associated with other cellular functions such as glucose uptake and cell differentiation and proliferation, and is associated with the glomerular mesangial cell proliferation and ECM deposition in early DN.42 In this study, we have demonstrated that p38 was activated in DN, which could be caused by miR-23b downregulation and increased G3BP2 expression. We have further shown that inhibition of the p38MAPK signal by SB203580 could decrease FN production in HK-2 cells exposed to high glucose (Figure 2E). We also found that miR-23b is regulated by G3BP2, P38MAPK, and the P53 cascade, possibly through a miR-23b/G3BP2 feedback circuitry. Thus, we speculate that a central mechanism for miR-23b is the inhibition of ECM protein production in DN through its action on p38MAPK. To the best of our knowledge, this is the first report showing miR-23b’s and its target G3BP2’s involvement in the pathogenesis of DN. Thus, miR-23b and G3BP2 may be therapeutic targets for DN.

CONCISE METHODS

Cell Culture
We obtained HK-2 cells derived from the proximal tubule and HRGE cells from American Type Culture Collection (ATCC), Manassas, VA. We purchased mouse podocytes from Peking Union Medical College Basic Medical Sciences Cell Resource Center. We treated the cells with normal glucose (5 mmol/L D-glucose), high glucose (25 mmol/L D-glucose), or with osmotic control (25 mmol/L L-glucose) for 24 hours.
Figure 6. Inhibition of miR-23b in normal mice promoted kidney fibrosis and increased urine protein excretion. (A–C) Representative images of PAS and FN staining of glomeruli (first panel) and renal tubules (second panel, inset image is augmentative tubules) in normal C57BL/6J mice after miR-23b antagomir (Anti–miR-23b) injection for 1 month (n=4). (D) Quantification of mRNA expression levels of various markers of fibrosis in normal mouse kidneys after miR-23b antagomir injection. (E) Electron microscopic analyses of the podocyte.
miRNA Mimic or Antagomir Transfection
We transfected HK-2 cells with miRNA-23b mimic (100 nmol/L; Rabio Co., Guangzhou, China) using Lipofectamine 2000 (Life Technologies, Carlsbad, CA), according to the manual. For mir-23b antagonir transfection, we directly added mir-23b antagonir (200 nmol/L) into HK-2 cells exposed to normal glucose. Scrambled controls were used in parallel.

Animal Experiments
The Mudanjiang Medical University animal care and veterinary services approved all protocols. The investigations conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. We obtained male C57BL/6 mice (9 weeks old) and male db/db mice (10 weeks old) from the SLAC Laboratory (Shanghai, China). We fed the animals on a standard rodent diet and water ad libitum. We induced type 1 diabetes by intraperitoneal injections of streptozotocin (in citrate buffer [pH=5.6]; three 70 mg/kg consecutive injections on alternate days). We defined diabetes as a blood glucose level >17 mmol/L on 2 consecutive days, and we euthanized the animals after 3 months of diabetes. One group of db/db animals received weekly intravenous injections by tail vein of 2.5 mg/kg G3bp2 agonir for 1 month.43 Likewise, another group of db/db animals received weekly intravenous injections of 2.5 mg/kg G3bp2 siRNA, and an altered group (control group) received the same dose of scrambled miRNA or siRNA in the tail vein, as previously described.43 We obtained all miRNAs and scrambled siRNAs from Rabio Co.. In addition, we intravenously injected normal male C57BL/6 mice by tail vein for 3 consecutive days with 80 mg/kg of mir-23b antagonist. We euthanized the animals at the end of 1 month of injections. We centrifuged the blood from the various mice at 5000 rpm for 10 minutes, and collected the serum. We performed an ELISA to measure insulin levels using a commercially available kit for the mouse (Merck GmbH, Darmstadt, Germany) according to the manufacturer’s instructions. We collected and stored kidneys and other tissues at −80°C.

miRNA Extraction
We performed miRNA extraction from human serum as previously described,44,45 and quantified miRNA expression levels by real-time PCR. This study was approved by the Ethics Review Board of Mudanjiang Medical University, Heilongjiang, China. We obtained informed consent from all patients. The patients’ clinical information is presented in Supplemental Table 2. We extracted miRNAs from the cells and tissues using the OMEGA RNA isolation kit (OMEGA Bio-Tek Inc., Norcross, GA). For serum extraction, we added three volumes of TRIzol solution (Life Technologies) to one volume of serum, vortexed this, and then incubated it at room temperature for 5 minutes. We added 5 μl of 20 pM ath-miR-156a mimic to all samples to provide a normalized control, then vortexed each sample immediately. We separated aqueous and organic phases by adding 0.2 volumes of molecular-grade chloroform. After vortexing at the maximum setting for 30 seconds, we centrifuged at 12,000 rpm for 15 minutes at 4°C. We rapidly transferred the aqueous phase to a new tube, and continued the TRIzol protocol. Finally, we dissolved total RNA in 15 μl of RNase-free water.

biRNA Extraction and Real-Time PCR
We extracted total RNA from cells and tissues using TRIzol reagent, according to the manufacturer’s instructions. The ribosomal protein S16 mRNA level served as the internal control. The primer sequences we used are listed in Supplemental Table 3.

Western Blot and ELISA Analysis
We homogenized the cells and tissues in radioimmunoprecipitation assay lysate (HaiGene, Harbin, China) containing protease inhibitor (Roche Hong Kong Limited, Hong Kong, China). We quantified total proteins using the bicinechonic acid assay kit (Galen Biopharm International Co. Ltd., Beijing, China). Samples (20 μg/lane) were resolved by SDS-PAGE, blotted, and probed with the following primary antibodies: anti-G3BP2 (Abcam Hong Kong Ltd., Hong Kong, China), anti-p38 or phosphorylated p38 (Cell Signaling Technology, Danvers, MA), and anti–β-actin (Abcam Hong Kong Ltd.). We measured serum TGF-β1 of patients with diabetic nephropathy using ELISA kits (Proteintech, Chicago, IL) according to the manufacturer’s instructions.

Luciferase Reporter Assay
Targeting G3BP2 3′-UTR
A G3BP2-3′-UTR luciferase vector including two different 3′-UTRs of G3BP2 (113 bp and 544 bp) containing the G3BP2-miR-23b response elements (wt1/2-Luc-G3BP2) and a mutant (mut1/2-Luc-G3BP2) lacking those elements were amplified by PCR from human cDNA. We cotransfected plasmid DNA (wt1/2-Luc-G3BP2, mut1/2-Luc-G3BP2, or β-galactosidase control vector) and mir-23b agonir into human embryonic kidney-293A cells for 48 hours. We measured luciferase activity using a SpectraMax M5 (Molecular Devices, Sunnyvale, CA) and normalized it by measuring β-galactosidase activity. The primers used to generate specific fragments for human G3BP2 3′-UTR are listed in Supplemental Table 4.
Histologic Analysis
We stained paraffin-embedded mouse kidney sections (5 μm) using the PAS kit (Abcam Hong Kong Ltd.) according to the manufacturer’s protocol. We used mouse monoclonal antibodies against G3BP2, FN, and collagen I and IV, and goat polyclonal secondary antibody against mouse IgG-H&L (Abcam Hong Kong Ltd.) for immunofluorescence analyses. For quantitative morphometry, we counted cells stained in ten randomly selected micrographs using Image-Pro Plus software (Media Cybernetics, Rockville, MD).

Ultrastructural Analysis
We fixed kidney tissues in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 24 hours, according to the manufacturer’s instructions. We used a JEM-1010 transmission electron microscope to visualize ultrastructures. From each specimen, we photographed and analyzed ten randomly selected areas using Image-Pro Plus software (Media Cybernetics).

Statistical Analyses
We analyzed data using a one-way ANOVA and a t test with Dunnett comparison using Prism (version 4; GraphPad Software, La Jolla, CA) and expressed them as the mean±SEM. We considered values significantly different if P<0.05.

ACKNOWLEDGMENTS
B.Z. designed the experiments and collected the data, contributed to the discussion, and reviewed and edited the manuscript. The other authors were involved in data collection.

This work was supported in part by the Natural Science Foundation of China (grant nos. 81102149, 81372951, and 81070329), the Heilongjiang Province Education Fund (nos. 81102149, 81372951, and 81070329), the Natural Science Foundation of China (grant nos. 11101143, 11101145, and 11401269), and the Heilongjiang Province Natural Science Foundation (H201496).

DISCLOSURES
None.

REFERENCES

www.jasn.org


This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2015030300/-/DCSupplemental.